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Award Number: W81XWH-04-1-0573

TITLE: BRCC36, a Novel Subunit of a BRCA1 E3 Ubiquitin Ligase Complex: Candidates for BRCA3

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REPORT DATE: June 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-06-2008	Final	15 MAY 2004 - 14 MAY 2008

4. TITLE AND SUBTITLE

BRCC36, a Novel Subunit of a BRCA1 E3 Ubiquitin Ligase Complex: Candidates for BRCA3

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0573

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Fox Chase Cancer Center
Philadelphia, PA 19111

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Breast cancer is a genetically heterogeneous disease, and multiple genes remain to be identified among BRCA1 and BRCA2 mutation-negative breast cancer-prone families. We hypothesized that other proteins, which have equivalent or complementary functions as BRCA1, may also be involved in the development of breast cancer. We have recently found one such candidate, referred to as BRCC36. We have reported a profound increase in BRCC36 expression in breast tumors which leads to tumorigenesis in vitro. Furthermore, our studies have defined BRCC36 as a direct regulator of BRCA1 activation and nuclear foci formation in response to IR in a number of breast cancer cell lines. Our results have found that down-regulation of BRCC36 expression impairs homologous recombination repair (HRR). Therefore, our data suggest that DNA repair pathway activated in response to IR and appears to sensitize breast cancer cells to IR-induced apoptosis. Importantly, we found that BRCC36 mutated in the germline of a cancer-prone family and may increase the risk of developing breast cancer. Overall, aberrant expression or mutation of BRCC36 genes in breast tumors may lead to disruption of the normal function of BRCA1 and contribute to the development of breast cancer.

15. SUBJECT TERMS
Breast cancer susceptibility, BRCA1, BRCA2, BRCC36, DNA damage/repair, tumorigenesis, and radiation sensitization.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	66	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

Breast cancer is the most common cancer affecting women, with a lifetime risk of breast cancer ~10% by the age of 80 years. In the United States, it has been estimated that there will be approximately 182,450 new cases of female breast cancer, and more than 40,480 breast cancer related female deaths in 2008 (Jemal, et al., 2008). It is estimated that 13.2 % of all American women (1 in 8) will develop breast cancer and 3.0 % will die from this disease (Ries, et al., 2007). Despite the advances in treatment and early detection, the mortality rate from breast cancer in women only decreased by 2.3 % per year between 1990 and 2002 (ACS, 2006). Current estimates suggest that family history is associated with 10-20 % of breast cancer in the United State according to studies by Collaborative Group on Hormonal Factors in Breast Cancer (2001). *BRCA1* (OMIM: 113705) and *BRCA2* (OMIM: 600185) are two of the most important breast cancer susceptibility genes, and deleterious mutations in these two genes account for about 15-30 % of familiar breast cancer (The Breast Cancer Linkage Consortium. 1999; Bove, et al., 2002; King, et al., 2003). Therefore, most familial aggregation of breast cancer remains unexplained. Furthermore, the majority of the tumors occur in women with little or no family history, and the molecular basis of these sporadic breast cancers is still poorly defined.

Identification of Novel Genes Involved in Breast Cancer Susceptibility

Extensive mutation testing has indicated that not all "high-risk" breast cancer families are due to mutations in *BRCA1* or *BRCA2* (Bove, et al., 2002; Ford, et al., 1998). However, the failure of linkage studies suggests that genes with penetrances similar to *BRCA1* and *BRCA2* are unlikely to exist, or at least that pathogenesis mutations in such genes will be very rare (Smith, et al., 2006). A more plausible hypothesis is that residual genetic susceptibility is driven by variants at many loci, each conferring a moderate risk of breast cancer (Antoniou and Easton, 2006). There is mounting evidence to support the speculation that there are additional, less prevalent breast cancer susceptibility genes (Antoniou, et al., 2001; Kainu, et al., 2000; Seitz, et al., 1997; Thompson, et al., 2002). Germline mutations of *TP53, PTEN, ATM, CHEK2, NBS1, RAD50, BACH1/BRIP1, and PALB2* are associated with breast cancer, but to a much more limited extent than *BRCA1* and *BRCA2* (Erkko, et al., 2007; Heikkinen, et al., 2006; Liaw, et al., 1997; Meijers-Heijboer, et al., 2002; Seal, et al., 2006; Steffen, et al., 2006; Thompson, et al., 2005; Walsh, et al., 2006). Therefore, compelling evidence suggests that other familial breast cancer genes exist, and new approaches must be used to identify new genes that predispose women (possibly men) to breast cancer.

A valid alternative approach is first to examine candidate genes in families with multiple cases of breast cancer, and next to determine whether such candidates carry mutations that might account for a proportion of these families with unknown genetic etiology. The most likely strategy is to evaluate genes that code for proteins with equivalent or complementary functions or function in the same pathway as BRCA1 and BRCA2, such as *CHEK2*, *BRIP1* and *PALB2*. Evidence is accumulating that dysfunction of other genes, coding for proteins in the same pathway as BRCA1 and BRCA2, might be important in the pathogenesis of a significant proportion of sporadic, non-familial breast cancer (Hughes-Davies, et al., 2003; Jazaeri, et al., 2002; Turner, et al., 2004). Therefore, this strategy may help to uncover important new insight into the role of BRCA1 and BRCA2 in sporadic breast and ovarian cancer, since *BRCA1* and

BRCA2 are rarely found mutated in these cancers (Futreal, et al., 1994; Lancaster, et al., 1996; Merajver, et al., 1995). One of the major problems of this strategy is that the complete function(s) of BRCA1 and BRCA2 have not been established, nor have the majority of the proteins which interact with BRCA1 and BRCA2 have not yet been identified.

BRCA1/2 Containing Complex (BRCC)

Much of the current scientific effort involving BRCA1 is being directed to defining the biochemical functions of BRCA1 and its interacting-proteins. Wang *et al* have previously reported that a set of proteins associate with BRCA1 to form a large mega-Dalton protein complex, referred to as BASC (BRCA1-Associated Genome Surveillance Complex) (Wang, et al., 2000). This complex includes several DNA damage repair proteins, MSH2, MSH6, MLH1, ATM, and the MRE11-RAD50–NBS1 protein complex. BASC is responsive to double stranded breaks (Wang, et al., 2000). However, it becomes clear that BRCA1 and/or BRCA2 can exist in a number of protein complexes and that many BRCA1/2 associated proteins remain to be identified.

Using a combination of affinity purification of anti-FLAG and mass spectrometric sequencing, we have reported a novel multiprotein complex, termed BRCC (BRCA1/2 Containing Complex) (**Figure 1**), which contains seven polypeptides including BRCA1, BRCA2, BARD1 and RAD51 (Dong, et al., 2003). We first reported that BRCC was an E3 ubiquitin ligase complex exhibiting activities in the E2-dependent ubiquitination of the tumor suppressor p53. In this multiprotein complex, three proteins, referred to as BRCC36, BRCC45, and BRCC120 have been found to be associated with BRCA1 and BRCA2.

Mass spectrometric sequence analysis of the 36-kDa, 45-kDa, and 120-kDa bands in **Figure 1** identified these proteins referred to as BRCC36, BRCC45, and BRCC120 (Dong, et al., 2003). The *BRCC36/c6.1A* gene is located at the Xq28 locus, a chromosomal break point in patients with prolymphocytic T-cell leukemia (T-PLL) (Fisch, et al., 1993). The chromosomal break occurred in two different introns of *BCC36/c6.1A* and the fusion transcripts were expressed at high levels in the leukaemic cells from T-PLL patients (Fisch, et al., 1993). In addition, BRCC36 bears sequence homology with both human Poh1/Pad1 subunit of the 26S proteasome and subunit 5 (Jab1) of the COP9 signalosome (Dong, et al., 2003). Our previous study has shown that a recombinant four-subunit BRCC complex containing BRCA1-BARD1-BRCC45-BRCC36 revealed an enhanced E3 ubiquitin ligase activity compared to that of BRCA1-BARD1 heterodimer (Dong, et al., 2003). As proposed in my fellowship grant, we plan to thoroughly establish the role of these BRCA1-associated proteins, especially, BRCC36, in the pathogenesis of both sporadic and familial forms of breast cancer.

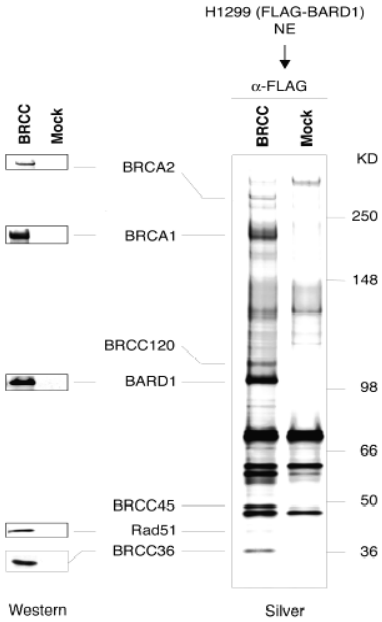

Figure 1. Purification of BRCC. Analysis of anti-FLAG eluate by SDS-PAGE followed by silver staining (Dong et al, 2003).

BODY

Task 1: To expand the evaluation of the expression of BRCC36 in clinical breast tumor samples, and to determine if over-expression of BRCC36 is associated with gene amplification in breast cancer.

Aberrant Expression of *BRCCs* in Majority of Sporadic Breast Cancer

The expression levels of *BRCC36* mRNA, analyzed by RT-quantitative PCR (RT-qPCR), were elevated in 58% (11 of 19) of the breast tumors evaluated in comparison with both normal breast organoids and primary epithelial cells (**Figure 2A**). A subset of these tumors (6 of 19) expressed extremely high levels of *BRCC36* relative to both the purified mammary organoids and primary epithelial cells. The difference in *BRCC36* mRNA levels in the normal organoids or primary epithelial cell cultures may be due to differences in age, parity, or hormonal status of the patients from whom the organoids were derived or to the tissue-culturing conditions of the primary epithelial cells. To further validate the expression of *BRCC36* in breast tumors, we performed qPCR analysis on laser capture microdissection (LCM)-purified normal mammary ductal epithelium (NE) cells and malignant epithelial (ME) cells. Two normal breast tissues and nine invasive ductal carcinomas were microdissected, and the mRNA expression was evaluated by RT-qPCR (**Figures 2B** and **2C**). We found that 100% of these tumors showed elevated levels of *BRCC36* mRNA relative to the normal mammary ductal epithelium (**Figure 2B**). The vast majority (~78%) of these tumors expressed very high levels of *BRCC36* (>20-fold) as compared to normal epithelial cells. To further demonstrate that BRCC36 is overexpressed in breast tumors, we evaluated protein expression using a BRCC36 polyclonal antibody. As shown in **Figure 2D**, the vast majority of tumor expressed significantly higher levels of BRCC36 related to adjacent normal tissues.

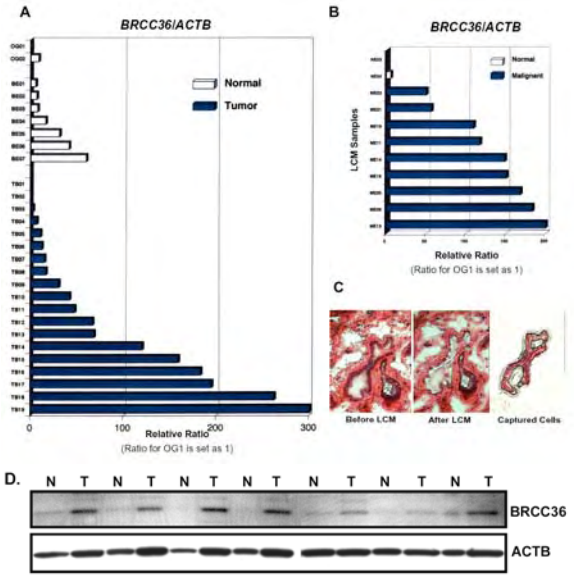

Figure 2. Overexpression of BRCC36 in Normal and Tumor Breast Samples. (A) QPCR was used to analyze breast mammary organoids (OG), primary breast epithelial cells (BE), and breast tumors (TB). **(B)** QPCR was utilized to analyze normal mammary ductal epithelial cells (NE) and malignant epithelial (ME) cells captured by laser microdissection (LCM). **(C)** Example of a normal mammary duct isolated by LCM procedure. (**D**) Western blot analysis of BRCC36 expression in normal and breast cancer tissues. For comparison, each paired normal tissues (N) and tumor tissue (T) obtained from same patient are arranged next to each other.

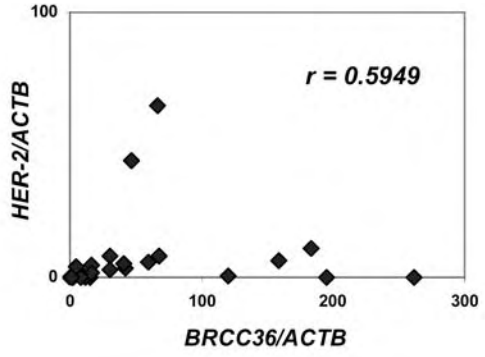

Figure 3. Correlation between *HER*-2 and *BRCC36* mRNA Expression in Breast Tumors. QPCR was utilized to analyze the target gene expression in breast tumors. The mRNA expression of *BRCC36* and *HER-2* were normalized by β-Actin (ACTB).

In addition,, after comparing the HER-2/neu mRNA expression in the same set of breast tumor samples, only 16% (3/19) of these breast tumors showed *HER-2/neu* amplification/over-expression

and no significant correlation between *BRCC36* and *HER2*/*neu* expression was found (Pearson correlation coefficient, r=0.5949; $p>0.2$) (**Figure 3**). This finding indicates that the aberrant expression of *BRCC36* is independent to *HER*-2/*neu* status in breast tumors. In addition, we have demonstrated that BRCC36 interacts with BRCA1 in MCF-7 or IR-exposed MCF-7 (**Figure 4**). We have also evaluated the expression of *BRCC45* and *BRCC120* in same set of the tumor and normal tissue samples. In comparison with normal breast organoids, the expression levels of *BRCC120* mRNA were elevated in 53% (10/19) of the breast tumors. In contrast, the expression levels of *BRCC45* mRNA were decreased in 32% (6/19) of breast tumors ($p<0.05$).

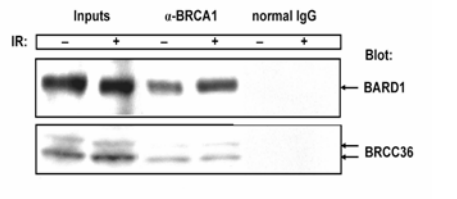

Figure 4. **Direct Interactions between BRCC36 and BRCA1**. 0.5 mg lysates from MCF-7 cells (w/o or with IR exposure) were incubated with anti-BRCA1 antibody or normal IgG (IP control). Immunoprecipitates were separated by SDS-PAGE, and the levels of BRCC36 were determined by anti-BRCC36 and anti-BARD1 (positive control) antibodies.

Studies of *BRCC36* Gene Amplification in Breast Tumors

Because *BRCC36* is located at chromosome X, we sought to examine if *BRC36* gene amplification is related to male breast cancer in a pilot study. Dual hybridization with a *BRCC36* BAC (RP11-143H17) probe and an X-chromosome alpha-satellite probe (CEP X, Vysis.) was performed in an identical way for eight male breast tumor specimens. After DAPI staining, FISH sections were then examined on an epifluorescence microscope. *BRCC36* gene amplification was defined as a mean *BRCC36* gene copy number to mean X-chromosome copy number ratio of greater than 1.5. In this small pilot study, *BRCC36* gene amplification has not been identified. However, one male breast tumor has been found to be hyperploids (**Figure 5**)

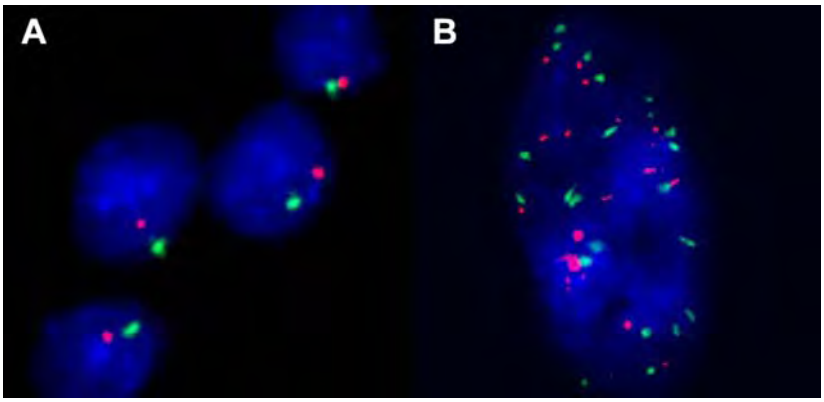

Figure 5. FISH Analysis of *BRCC36* Amplification in Male Breast Carcinoma. (**A**) BRCC36 (green) and X-chromosome alpha-satellite probe (red) were present in normal copy number. (**B**) BRCC36 and centromere X were present in abnormal copy number. The cell was counterstained with DAPI (Blue).

Task 2: To determine the frequency of BRCC36 germline mutation in BRCA1 and BRCA2 mutation negative breast cancer prone kindreds.

Mutation of *BRCC36* in a Breast Cancer-Prone Kindred

To examine *BRCC36* gene for germline mutations, we selected breast cancer-prone kindreds that have tested negative for *BRCA1* and *BRCA2* germline mutations through the Family Risk Assessment Program. A set of 11 oligonucleotide primer pairs were designed to amplify genomic DNA region for the *BRCC36* coding exons. Blood DNAs from probands affected with breast cancer and reporting at least 2 first-degree and/or second-degree relatives with breast cancer were evaluated for germline mutations. In the initial screen, a frameshift *BRCC36* mutation (c.880insGGGGdup148) was identified in the germline of a *BRCA1/2* mutation-negative breast cancer prone kindred when evaluating exon 11 (**Figures 6A-C)**. The proband (II-1) was diagnosed with bilateral breast cancer at age 48 (designated by the arrow). We were not able to test her father (I-2, BCC at 61 and colon cancer at 81) and one of her sisters (II-3, early-onset breast cancer at age 32) since they died before specimens could be obtained. However, both of these individuals are the obligate carriers because proband's mother (I-1) did not carry this mutation and *BRCC36* is located on chromosome X. The proband's other sister (II-2, bilateral breast cancer at age 53) also carried this mutation. In addition, the proband reported a niece (III-4) with early-onset melanoma, and her DNA sample was positive for

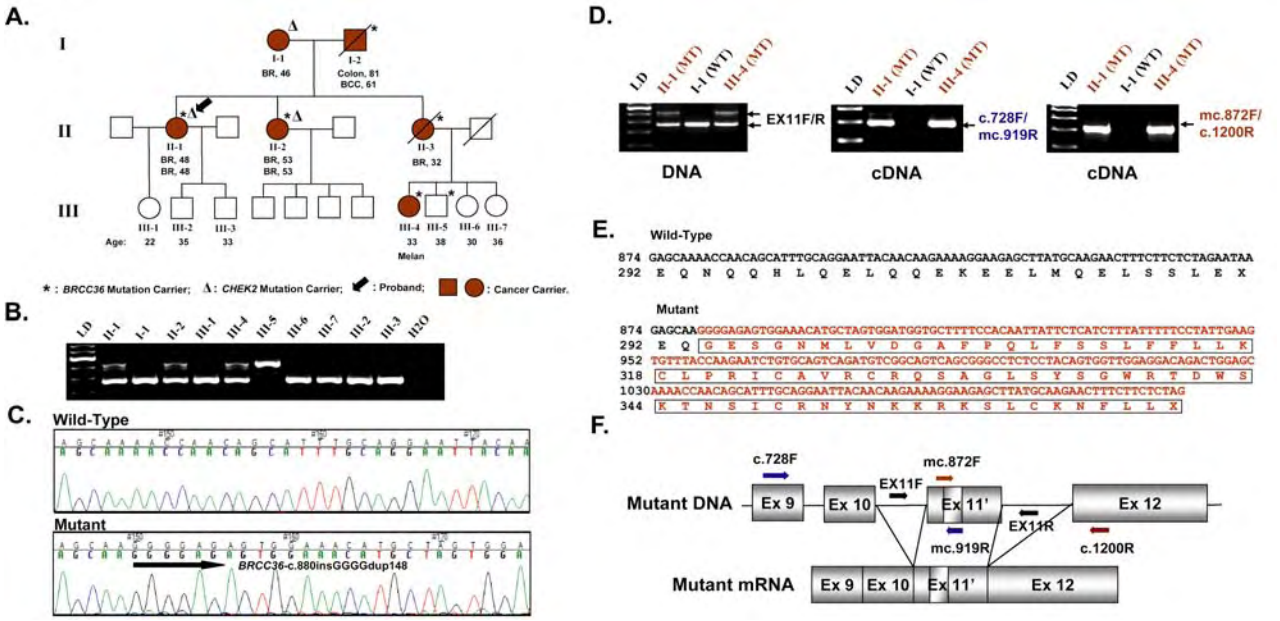

Figure 6. A *BRCC36* Frameshift Mutation *(*c.880insGGGdup148). (**A**) Pedigree of a breast cancer prone kindred with a *BRCC36* frameshift mutation (c.880insGGGdup148). (**B**) Agarose gel image of PCR products of exon 11 for DNAs isolated from the peripheral blood of affected and unaffected family members. Samples from left to right are DNA ladder, PCR products from individuals and a negative control (H_2O). (**C**) Sequencing analysis for I-1 (wild type) and III-5 (homozygote *BRCC36* c.880insGGGGdup148 mutation carrier. (**D**) Agarose gel image of PCR products of *BRCC36* exon 11 from genomic DNAs (left panel) and cDNAs (middle and right panel isolated or synthesized (RT) from immortalized LCLs, respectively. Samples from left to right are DNA ladder, PCR products from LCLs, II-1 (mutant), I-1 (wild type), and III-4 (mutant). Primer sets used for each PCR reaction were listed with the gel image. (**E**) Protein sequence analysis of *BRCC36* c. 880insGGGGdup148 mutation. This frameshift mutation is predicted to result in expression of a mutant protein [i.e., 72 new residues beginning at 294 and a stop codon at residue 366 (p.Arg294ThrfsX73)]. (**F**) Schematics for the splicing of the *BRCC36*-c.880insGGGGdup148 allele.

carrying this *BRCC36* mutation. Interestingly, we also identified a *CHEK2*-c.1100delC mutation in proband (II-1), her mother (I-1), and her sister (II-2). Inactivating mutations in low-penetrant breast cancer susceptibility alleles, such as *CHEK2* and *ATM*, are associated with modest risks (approximately two-fold) as compared to *BRCA1* or *BRCA2* mutations (10 to 20-fold by age 60) (Meijers-Heijboer, et al., 2002; Thompson, et al., 2005). Therefore, these results suggest that *BRCC36* and *CHEK2* may be cooperating to confer susceptibility to breast cancer in compound heterozgotes.

To determine if the *BRCC36* frameshift mutation results in expression of a mutant transcript, we derived EBV immortalize lymphoblastoid cell lines (LCLs) using lymphocytes collected from the proband (II-1: mutation carrier), her mother (I-1: wild-type), and her niece (III-4: mutation carrier). The protocol for immortalizing lymphocytes has been described previously (Chen et al, Human Mutation, 2006, in appendix). Analysis of genomic DNA by PCR confirms that LCLs from II-2 and III-4 carry this mutation, while the I-1 line is wild-type (**Figure 6D**). Total cellular RNAs were then isolated to perform RT-PCR with primers specifically targeting *BRCC36*-c.880insGGGGdup148 mutant transcripts. As shown in **Figure 6D**, the *BRCC36*-c.880insGGGGdup148 allele is expressed clearly in II-1 and III-4, but not in I-1 LCLs. Following sequencing analysis, a mutant exon 11 (Ex 11'), with an additional 152 nucleotides incorporated, was identified (**Figures 6E** and **F**). Because this frameshift mutation affects the sequence of the C-terminal last 23 amino acids of the BRCC36 protein, the original BRCC36 antibodies, which were derived against the last 20 amino acids, cannot be used to detect the mutant form. We are currently evaluating series of antibodies derived against amino acids 40-53 and 176-189.

BRCC36 Isoforms

In addition, an intron alteration (IVS2-29delT) was identified in one primary breast tumor. During the process of amplifying various regions of the cDNA, we identified one splicing variant of *BRCC36* (**Figure 7**). This alternative splicing leads to in-frame 25 amino acids insertion. The functional significance of this variant is yet clear.

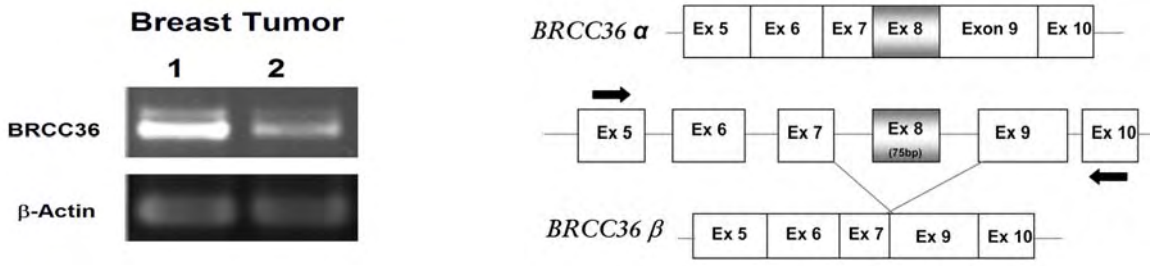

Figure 7. Splicing Variants of *BRCC36* in Breast Tumor. Left panel, RT-PCR analysis by agarose gel image. Right panel: schematics for *BRCC36* alternative splicing. Sequencing analysis for PCR samples was performed using TOPO cloning.

Task 3: To determine the role of BRCC36 in tumorigenesis by studying if its over-expression contributes to malignant transformation of mammary epithelial cells.

Inhibition of *BRCC36* Gene Expression by siRNA in Breast Cancer Cell Lines

To further elucidate the functional consequence of *BRCC36* aberrant expression in the pathogenesis of breast cancer, we performed *in vivo* silencing studies targeting *BRCC36* in MCF-7, T47D, and ZR-75-1 breast cancer cell lines, which constitutively express high levels of *BRCC36* transcript relative to non-tumorigenic breast epithelial cells, i.e., MCF-10F, MCF-12A (**Figure 8A**). Since antibodies specific to BRCC36 protein are not available, quantitative PCR was used to establish constitutive and attenuated *BRCC36* mRNA levels. We used siRNA targeting *BRCC36* or GFP (negative control) to assess the response of *BRCC36* depletion in breast cancer cells. All of the siRNA duplexes were synthesized by Dharmacon using 2'-ACE protection chemistry. Treatment with this siRNA resulted in a >50-80% decrease in BRCC36 mRNA levels in comparison to mock treated or siRNA-control transfected cells ($p<0.05$) by 72 hrs. The greatest level of suppression was observed in MCF-7 cells (**Figure 8B**).

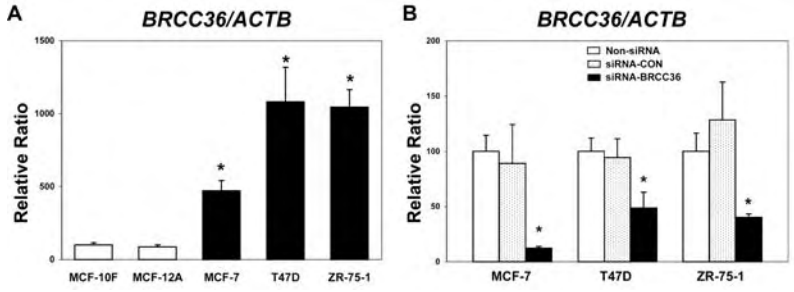

Figure 8. Abrogation of *BRCC36* Expression by siRNA Treatment. The relative level of *BRCC36* expression was adjusted with β-actin (ACTB). **(A)** *BRCC36* expression in non-tumorigenic (MCF-10F, MCF12A) and tumorigenic (MCF-7, ZR-75-1, and T47D) breast epithelial cell lines. **(B)** *BRCC36* expression in breast cancer cell lines after siRNA treatment ($p<0.05$).

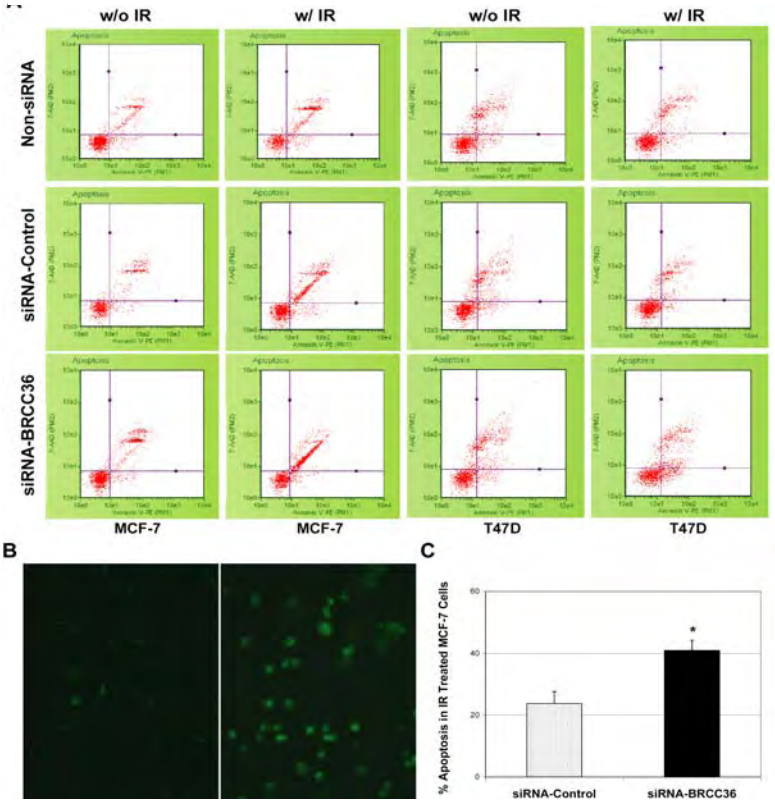

Figure 9. Apoptosis Analysis in Breast Cancer Cells Exposed to IR. (A) MCF-7 and T47D cells were mock treated (non-siRNA) or were transfected with siRNA-control or siRNA-*BRCC36* prior to IR exposure. The proportion of apoptotic cells was measured following annexin V and 7-amino actinomycin D staining using a Guava Personal Cytometer. **(B)** TUNEL labeling was performed to detect apoptotic MCF-7 cells (light green) following exposure to IR. **(C)** Data analysis of the TUNEL assay. At least 1200 cells of each treatment group from 8 independent fields were counted to evaluate the percentage of apoptotic cells.

Depletion of *BRCC36* Enhanced IR-Induced Breast Cancer Cell Apoptosis

Since the BRCA1 protein is activated via the ATM/CHK2 signaling pathway following the exposure of cells to DNA damaging agents such as ionizing

radiation (IR) (Scully, et al., 1997), we sought to determine the role, if any, of BRCC36 in this response. Following depletion of *BRCC36* via siRNA, MCF-7, ZR-75-1, or T47D were irradiated utilizing a Cesium 137 Irradiator (Model 81-14R). The cells received 4-Gy total IR (1.132 Gy/min for 3.53 mins) for a targeted 50% induction of apoptosis (Liebmann, et al., 1994). The cells were cultured for an additional 72 hours prior to harvesting and were examined for DNA damage-induced cell apoptosis via Annexin V and 7-amino actinomycin D staining. The proportion of apoptotic cells was determined utilizing a Guava Personal Cytometer (Guava Technologies) according to the manufacturer's instruction. No significant difference in the fraction of cells undergoing apoptosis in mock treated, siRNA-control transfected, or siRNA-*BRCC36* transfected cells was observed in the absence of IR, indicating that depletion of BRCC36 alone is not lethal (**Figure 9A**). However, when combined with *BRCC36* knock-down, IR exposure led to a significant increase in the percentage of MCF-7 cells that undergo apoptosis (45.9% ± 4.3%) when compared to the siRNA control group (34.9% ± 1.9%, $p<0.05$) (**Figure 9A and Table 1**). Consistent with these results, the overall cell viability was substantially lower in siRNA-*BRCC36* treated cells following IR as compared to control cells (50.9% ± 5.8% vs. 58.4% ± 5.7%) (**Table 1**). Similar results were observed in T47D cells treated with siRNA-BRCC36 and IR versus controls (42.2% ± 4.5% vs. 23.3% ± 1.9%, $p<0.05$) (**Figure 9A and Table 1**). Although the trend was evident for ZR-75-1 cells, the fraction of cells undergoing apoptosis following depletion of *BRCC36* and IR were not statistically significantly different (**data not shown**). Induction of apoptosis was confirmed using a TUNEL assay and MCF-7 cells (**Figure 9B**). The combination of *BRCC36* siRNA abrogation and IR exposure again resulted in a significant increase in the fraction of cells undergoing apoptosis when compared to the siRNA-control treated cells (40.9% ± 2.7% vs. 24.9% ± 3.3%) ($p<0.05$) (**Figure 9C**). As a result, BRCC36 depletion appears to substantially sensitize breast cancer cells to IR-induced apoptosis.

Table 1. Nexin Assay in Breast Cancer Cells Exposed to IR

	MCF-7		T47D	
	Viable Cells	Apoptotic Cells	Viable Cells	Apoptotic Cells
Non-siRNA	57.3% ± 7.2%	34.4% ± 4.5%	68.5% ± 0.3%	26.5% ± 0.4%
siRNA-Control	58.4% ± 5.7%	34.9% ± 1.9%	70.5% ± 2.3%	23.3% ± 1.9%
siRNA-BRCC36	50.9% ± 5.8%	45.9% ± 4.3%†	52.7% ± 2.4%	42.2% ± 4.5%†

†:siRNA-BRCC36 vs. siRNA-Control (Student's *t*-test, $p<0.05$).

Inhibition of BRCC36 Disrupted BRCA1 Phosphorylation Induced by IR

Previous studies have indicated that the BRCA1 protein is phosphorylated in response to DNA damaging agents (Scully, et al., 1997). Since BRCC36 directly interacts with BRCA1 (Dong, et al., 2003), we examined the effect of *BRCC36* depletion on BRCA1-associated DNA repair/damage pathways. MCF-7 cells were treated with siRNA targeting *BRCC36*, and then exposed to 4-Gy of IR to induce DNA damage. MCF-7 cells were harvested at two hours after IR. Western blot analysis was carried out to examine the expression and modification of BRCA1, p21, p53, and ATM. Western

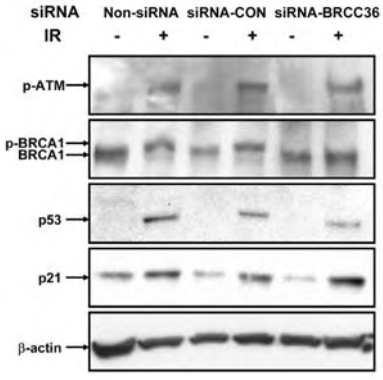

Figure 10. Activation of BRCA1 in Response to IR Treatment. MCF-7, MCF-7/siRNA control and MCF-7/siRNA-*BRCC36* cells were treated with or without IR (4-Gy), and cells were evaluated at 2h after radiation exposure. BRCA1 protein was evaluated by immunoblotting with anti-BRCA1 antibody and shifts in the mobility of the protein bands indicated phosphorylated and unphosphorylated protein. The protein levels of phosphorylated ATM, p53, and p21 were determined by immunoblotting with anti-p-ATM, anti-p53, and anti-p21 antibodies, respectively. Protein loading levels were evaluated by immunoblotting with anti-β-actin antibody.

analysis clearly showed that DNA-damage induced by IR resulted in increased expression of p21, stabilization of p53, and phosphorylation of BRCA1 and ATM (S1981) as expected (**Figure 10**). This same pattern was observed in siRNA-Control treated cells. Importantly, reduction of BRCC36 blocked IR-induced phosphorylation of BRCA1. In comparison, BRCC36 knock-down had no effect on IR-induced expression of p21, stabilization of p53, and phosphorylation of ATM.

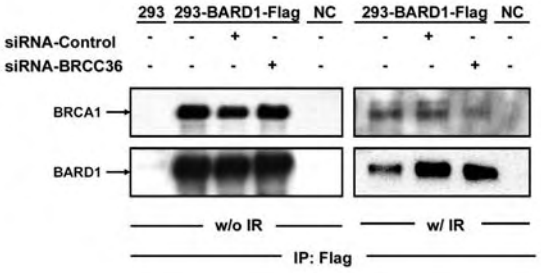

Figure 11. Effects of Inhibition of BRCC36 on the Integrity of BRCA1-BARD1 Heterodimer. 293-BARD1-FLAG cells were transfected with either siRNA-*GFP* (siRNA control) or siRNA-*BRCC36*. Transfected cells were then treated with 4-Gy IR and were incubated for 2 hours before harvesting. 1.5 mg of 293 cell lysate (control or BRCC36-siRNA transfected) was incubated with ANTI-FLAG M2-Agarose Affinity Gel. The protein levels of BRCA1, and BARD1 were determined by immunoblotting with anti-BRCA1 and anti-BARD1 antibody, respectively (NC: negative control).

Effects of Inhibition of BRCC36 on Integrity of BRCA1-BARD1 Heterodimer

To determine if depletion of BRCC36 affects the integrity of the BRCA1-BARD1 heterodimer, we targeted *BRCC36* mRNA in a 293-derived cell line expressing FLAG-BARD1 (Dong, et al., 2003). Immunoprecipitation was performed utilizing anti-FLAG on lysates prepared from cells transfected with the BRCC36 or control siRNAs. BRCA1 and BARD1 were examined with SDS-PAGE and western blot. Reduction of BRCC36 did not appear to alter the BRCA1-BARD1 interaction in either untreated or IR treated cells (**Figure 11**).

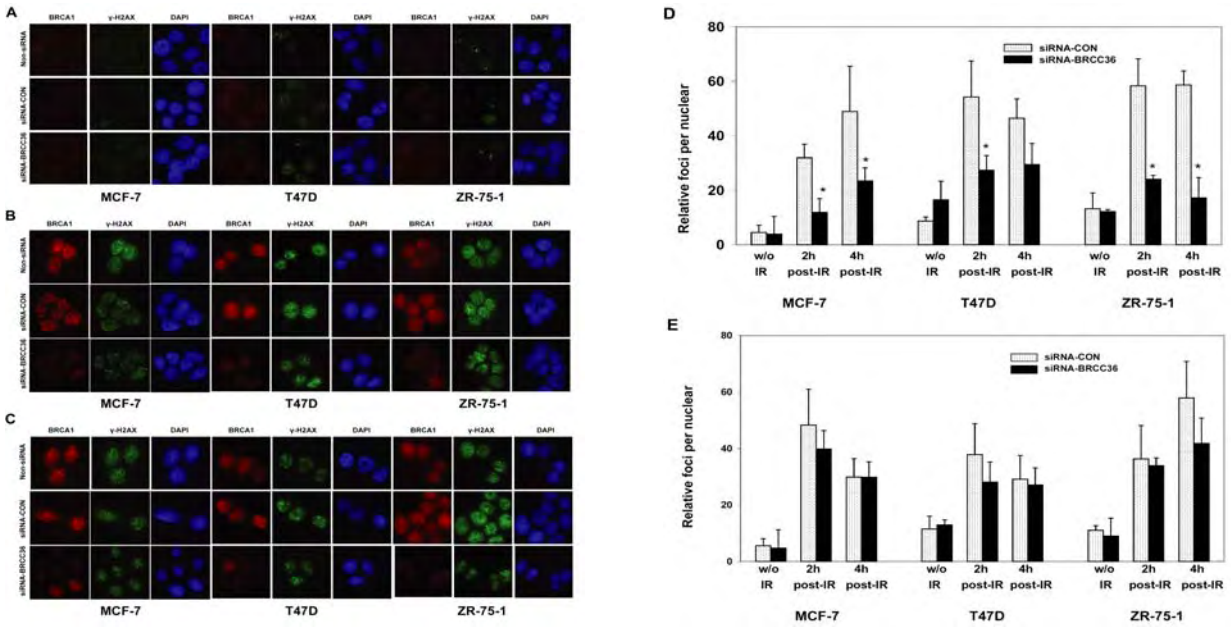

Figure 12. BRCA1 Nuclear Foci Formation in Breast Cancer Cells Following IR Exposure. Cells were mock treated (non-siRNA) or transfected with siRNA-CON or siRNA-BRCC36. Transfected cells were then treated with 4-Gy IR and were incubated for 2 or 4 additional hours. After pre-extraction and fixation, transfection cells then were immunostained for BRCA1 and γ-H2AX. Quantification of BRCA1 nuclear foci formation was performed with Metamorph® software (v6.1.). **(A)** BRCA1 and γ-H2AX nuclear foci formation without IR exposure. **(B)** BRCA1 and γ-H2AX nuclear foci formation at 2h post-IR exposure. (**C**) BRCA1 and γ-H2AX nuclear foci formation at 4h post-IR exposure. Quantification of BRCA1 (**D**) and γ-H2AX (**E**) nuclear foci formation without IR or at 2h and 4h post-IR exposure.

Inhibition of BRCC36 Disrupted BRCA1 Nuclear Foci Formation in Breast Cancer Cells Exposed to IR

It is well characterized that BRCA1 localizes to discrete nuclear foci (dots) during S phase or in response to DNA damage. In our previous report, we demonstrated that BRCC36 directly interacts with BRCA1 at the region encompassing amino acids 502-1,054 (Dong, et al., 2003). This region falls within the BRCA1 DNA-binding domain (a.a. 452-1079). Because the DNA-binding domain has been shown to contribute to the BRCA1 re-localization after DNA damage (Scully, et al., 1997; Wang, et al., 2000), we sought to evaluate the role of BRCC36 in the formation of BRCA1 nuclear foci in response to DNA damage. MCF-7, T47D, and ZR-75-1 cells, mock treated or transfected with siRNA-control or siRNA-BRCC36, were exposed to IR (4-Gy), and were evaluated for BRCA1 and γ-H2AX subcellular location. Recent studies have shown that the three breast cancer cell lines used in this study possess wild-type BRCA1 (Elstrodt, et al., 2006). As shown in **Figures 12A, B,** and **C**, BRCC36 deficiency inhibited BRCA1 focus formation as compared to mock treated and siRNA control transfected cells. Importantly, γ-H2AX response to IR was unaffected in the cells transfected with BRCC36 siRNA (**Figures 12A, B, C** and **E**). Quantification of BRCA1 nuclear foci showed that siRNA-BRCC36 transfection in MCF-7 cells resulted in 63% and 52% decrease compared with siRNA-control cells at 2h and 4h post-IR, respectively ($p<0.05$) (**Figure 12D**). Similar results were observed in T47D (49% and 36%) and ZR-75-1 (59% and 71%) cells (**Figure 12D**). Collectively, these results show that down-regulation of BRCC36 expression impairs the DNA repair pathway activated in response to IR by inhibiting BRCA1 activation.

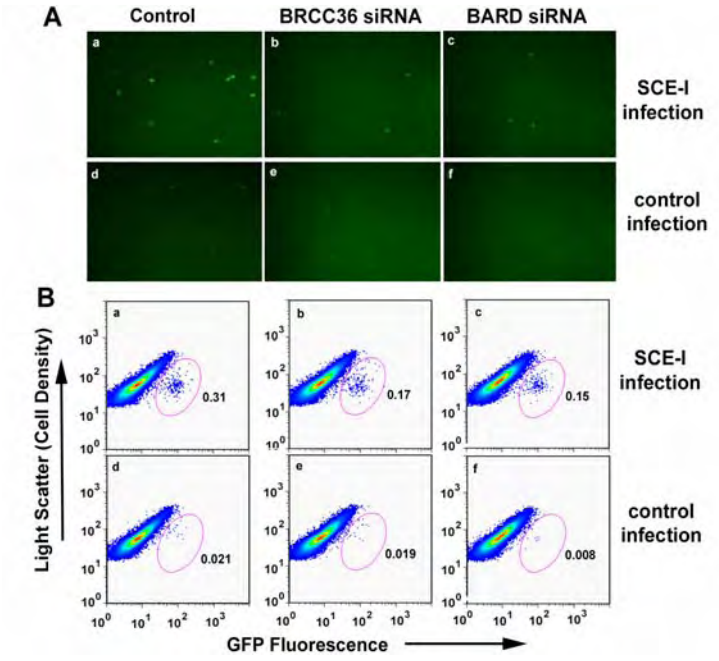

Figure 13. Depletion of BRCC36 Impairs Homologous Recombination Repair (HRR) in Cells Stably Transfected with the DR-GFP DNA Repair Substrate. (**A**) Fluorescent microscopy of DR-GFP cells transduced with lentiviral vector expressing SCE-I restriction enzyme (*top panels)* or with control "gutted" lentivirus *(bottom panels)* at 72 hrs post infection. Prior to infection, cells were transfected with siRNA-control (panels a & d), siRNA-BRCC36 (panels b & e), or siRNA-BARD1 (panels c & f) for 48 hours. (**B**) Flow cytometry of DR-GFP cells at 72h post infection. GFP-positive cells were gated (in the circular insert with percentage indicated) according to two-dimensional plot of GFP-specific fluorescence versus light scatter to identify GFP-positive cells.

Depletion of *BRCC36* Impairs Homologous Recombination Repair (HRR)

We have established a functional GFP-based assay to assess specific double-strand break repair. This assay is based on a reporter construct assay [modified from (Wang, et al., 2004)], where a plasmid containing a single 18bp I-SceI restriction site within an inactive green fluorescence protein (GFP) expression cassette is specifically cleaved allowing the detection of homologous recombination repair (HRR) by GFP expression. The pDR-GRP was kindly

provided by Dr. M. Jasin, Memorial Sloan Kettering (Pierce, et al., 1999). In addition, since the earlier submission we have generated a lentivirus construct (Lenti-SceI-NG) which contains the SceI endonuclease (Taghian and Nickoloff, 1997). In these preliminary studies, DSB repair can be detected at 12h post-infection with Lenti-SceI-NG, and repair reaches plateau levels between 48h and 72h (**Figure 13 panel A**). To observe the effect of BRCC36 and BARD1 on HRR of DNA DSBs, we combined the pDR-GFP-*Sce*I system and siRNA approaches. We treated DR-GFP cells with either BRCC36 and BARD1 siRNAs or control siRNA twice (at 0 and 48 hrs), and infected cells with SceI viral particles at 72h. Cells were collected for measuring the GFP signal at 72h after the infection. The results show that when BRCC36 and BARD1 expression were inhibited, the efficiency of HRR in these cells was lower than that in the control siRNA treated cells (**Figures 13A,** and **13B**), thus suggesting that BRCC36 and BARD1 are involved in HRR.

Silencing of BRCC36 Decreases the Expression of MRE11, a Subunit of the MRN Complex

The BRCA1-associated MRE11-RAD50-NBS1 (MRN) complex has recently been demonstrated to activate CHEK2 downstream from ATM in response to replication-mediated DNA double strand breaks (Takemura et al, JBC, 2006). To study the mechanism(s) of BRCC36-mediated activation of BRCA1 in response to the DNA damage, we first examined whether abrogation of BRCC36 affects the expression of MRE11, a subunit of the MRN complex. As we described previously (Chen, et al., 2006), HeLa cells were transfected twice with siRNA-BRCC36 or a siRNA-control. Twenty-four hours after the second siRNA transfection, cells were harvested and lysed for Western blot analysis. Results clearly showed that siRNA-BRCC36 treatment significantly knocked down BRCC36 protein levels (**Figure 14**). Importantly, although not affecting cell viability or the levels of CHEK2, depletion of BRCC36 by siRNA targeting significantly decreased the level of MRE11 compared to cells transfected with siRNA control (**Figure 14**). These preliminary findings suggested that the abrogation of BRCC36 potentially interferes with the normal nuclear localization of BRCA1 observed during S phase or in response to DNA damage possibly by mediating the integrity of the MRN complex. In addition, these data also suggest that MRN subunits may be potential substrates for BRCC complex.

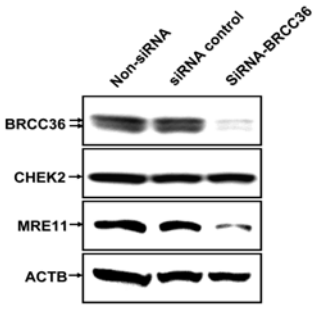

Figure 14. Silencing of BRCC36 down-regulates the expression of MRE11. HeLa cells were mock treated (non-siRNA) or were transfected with siRNA-control or siRNA-BRCC36. The expression of BRCC36, CHEK2, and MRE11 were determined by immunoblotting with specific antibodies. Protein loading levels were evaluated with anti-β-actin antibody.

To Functionally Characterize the Role of BRCC36 in BRCA1-Mediated Ubquitination

The BRCA1 N-terminal RING-finger region is the site of heterodimerization of BRCA1 and BARD1 (BRCAl-associated RING domain 1). When bound to BARD1, BRCA1 shows significant ubiquitin ligase activity and is capable of polymerizing ubiquitin. More importantly, deleterious mutations in the BRCA1 RING-finger domain abolish the ubiquitin ligase activity of BRCA1 (Hashizume, et al., 2001; Ruffner, et al., 2001). These findings suggest a relationship between BRCA1's ligase activity and the predisposition to breast cancer. At present, the known substrates that are polyubiquitinated by the BRCA1-BARD1 ubiquitin ligase are very limited. These known substrates include RNA polymerase II, nucleophosmin/B23, CtIP, and p53 (Dong,

et al., 2003; Sato, et al., 2004; Starita, et al., 2004). We have previously reported that reconstitution of a recombinant four-subunit BRCC complex containing BRCA1/BARD1/BRCC45/BRCC36 reveals an enhanced E3 ligase activity compared to that of BRCA1/BARD1 heterodimer (Dong, et al., 2003). In addition, BRCC36 displays sequence homology with the human Poh1/Pad1 subunit of 26S proteasome and with the subunit 5 (Jab1) of COP9 signalosome (Dong, et al., 2003). The COP9 complex has been shown to regulate the activity of the SCF ubiquitin ligase complex (Lyapina, et al., 2001; Yang, et al., 2002). In collaboration with Dr. R. Dunbrack (Director, Molecular Modeling Facility at FCCC), we have performed protein modeling analysis on isoform B of BRCC36. A multiple-round PSI-BLAST sequence search was performed using the BRCC36 amino acid sequences. Suitable templates available in the Protein Data Bank (PDB) were chosen by using the PSIBLAST profiles obtained in the first step (Berman, et al., 2000). The backbone and the conserved parts of the template were copied from the PDB structure and amino acids different from the target sequence were built using SCWRL3 (Canutescu, et al., 2003). The molecular visualization and three-dimensional structure manipulation were performed using Chimera (Huang, et al., 1996). The data from the protein structure analysis suggest that BRCC36 contains a Jab1/MPN domain metalloenzyme (JAMM) motif, and the conserved BRCC36 residues (His122, His124, Asp135 and Ser132) form a catalytic site that binds a Zn atom (**Figure 15**). By analogy with the catalytic mechanism involved by cytidine deaminase, it is suggested that the Zn ion activates a water molecule that is subsequently involved in a nucleophilic attack in the process of isopeptide bond hydrolysis. Furthermore, JAMM motif has been found to have isopeptidase activity in the proteins involved in neddylation and ubiquitination (Cope, et al., 2002; Tran, et al., 2003). These findings from BRCC36 protein modeling suggest that BRCC36 may play a direct role in ubiquitination.

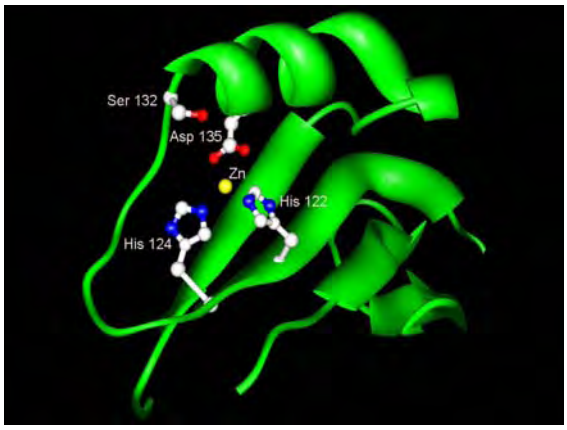

Figure 15. BRCC36 JAMM motif. Homology modeling procedures were used to obtain the three-dimensional model of isoform B of BRCC36. The molecular visualization and three-dimensional structure manipulation were performed using Chimera.

Proteome Analysis by 2D-gel between BRCC36-overexpression MCF-10A and Parental Lines

Our previous studies have shown that reconstitution of BRCA1-BARD1-BRCC45-BRCC36 reveals an enhanced E3 ubiquitin ligase activity when compared to that of BRCA1-BARD1 heterodimer (Dong, et al., 2003). A recent study has reported that BRCC36 displays deubiquitinating (DUB) activities towards K63-linked ubiquitin substrates (Sobhian, et al., 2007). In this capacity, BRCC36 has the potential to interact with numerous protein substrates and subsequently affect their stabilization, potentially explaining the possible oncogenic and tumor suppressor phenotypes associated with overexpression as observed in breast tumors or mutations found in hereditary diseases. We have initiated a pilot study to determine if BRCC36 can mediate protein stability using 2D-gel analyses. In this pilot study, MCF-10A cells were transfected with a GFP reporter plasmid and either BRCC36-flag or the control vector. Transfection efficiency was determined by eGFP and BRCC36 expression was determined by immunoblotting (**Figures 16A** and **B**). Forty-eight hours after transfection, cells were harvested

and lysed in 2D-buffer. Fifty microgram protein from BRCC36 or control vector transfected cell lysates was separated by IEF over a pH 3–10 range followed by gradient SDS-PAGE. After fixing and staining, the gels were scanned with ProXPRESS™ Proteomic Imaging System. Example images from one of triplicate comparisons are shown in **Figure 16C**. After analyzing by Progenesis (Nonlinear Dynamics, Inc.) (**Figure 16D**), the density of 22 spots was identified to be increased at least 3-fold in cells transfected with BRCC36 alone than those transfected with empty vector, while 9 spots were 3-fold lower in BRCC36-transfected cells ($p<0.05$). Since BRCC36 displays DUB activity, it is expected that the substrates of BRCC36 may be up-regulated when BRCC36 is overexpressed. However, based on our previous study and recent advancement (Dong, et al., 2003; Sobhian, et al., 2007), the DUB activities of BRCC36 may be dependent on which BRCA1 complexes BRCC36 participates in, and therefore, different BRCA1 complexes may either stabilize or promote degradation of their various substrates. Our preliminary 2D-gel analyses would suggest that both scenarios might be in effect following exogenous BRCC36 expression. Although 2D-gel protein analysis is limited to more abundant proteins, we are able to resolve 1000s of individual proteins and their isoforms. LC-MS/MS is being used to identify the protein spots consistently altered in repeated experiments through our Biotechnology Core Facility at FCCC to identify potential substrates of the BRCC complex or BRCC36.

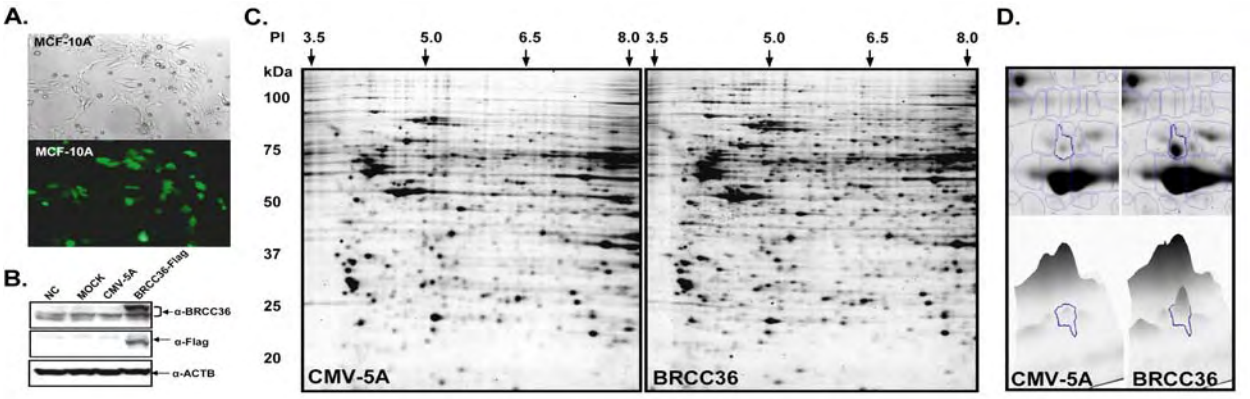

Figure 16. Two-dimensional Gels Analyses between BRCC36-overexpression MCF-10A and Parental Lines. (**A**) One million MCF-10A cells were electroporated with an eGFP construct and either 2 µg of a control plasmid (i.e., pFLAG-CMV2-5a) or a pFLAG-CMV2-BRCC36 plasmid via Nucleofector kit V (Amaxa). Transfection efficiency was determined to be ~60% using eGFP as a marker. (**B**) The expression of BRCC36 and Flag were determined by immunoblotting with specific antibodies. Protein loading levels were evaluated with anti-β-actin antibody. (**C**) Two-dimensional gels were run using protein lysates from MCF-10A cell transfected with CMV-5a empty vector (left) or BRCC36-Flag (right). Molecular weight markers are indicated in kDa and approximate isoelectric point is indicated across the top of the gels. (**D**) An example of 2D-analyses using Progenesis software: one protein (MW: ~60; PI: ~4.5) has been found to be overexpressed more than 3-fold in MCF-10A cell transfected with BRCC36 in comparison to the cell transfected with control vector.

BRCC36 Overexpression Stimulates the Colony Formation in a hMEC line, MCF-10F

We have evaluated whether exogenous expression of BRCC36 alone could transform MCF-10F, a non-tumorigenic epithelial cell line, by assessing anchorage-independent growth. As shown in **Figure 17**, FLAG-tagged BRCC36-overexpressing MCF-10F cells formed more colonies (>30 cells) in soft agar, as compared to vector-control MCF-10F cells. After quantification, BRCC36-overexpressing MCF-10F cells are ~5-times more efficient in colony formation than vector-control cells (18.5 ± 2.1 vs. 3.5 ± 0.7, $p< 0.01$) (**Fig. 17B**). In addition, since the transfection efficiency and the level of exogenous BRCC36/cell were not determined in

these initial experiments, it is possible that the additional experiments planned will yield an even more robust transformed phenotype as seen for MCF-7 cells (positive control, **Fig. 17C**). Therefore, we are currently deriving a BRCC36 expression vector (pBMN-I-GFP backbone) in which transfected cells can be sorted for GFP by FACS (bicistronic BRCC36/GFP mRNA) prior to plating in soft agar.

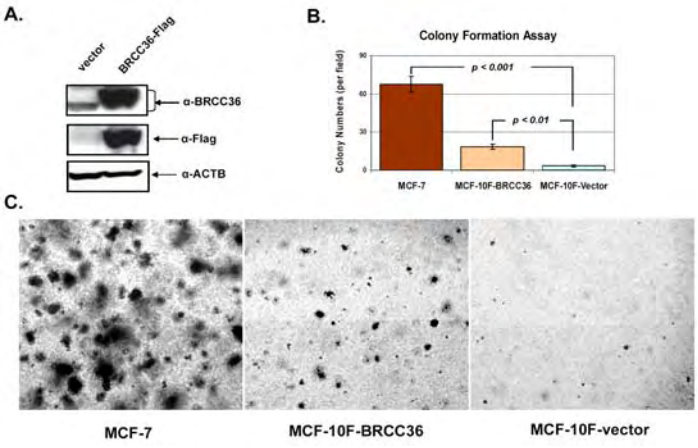

Figure 17. Exogenous Overexpression of BRCC36 Stimulates Colony Formation in MCF-10F Cells. (**A**) The expression of BRCC36 in MCF-10F cells transfected with either a control vector (pFLAG-CMV2-5a) or a BRCC36-expression vector (pFLAG-CMV2-BRCC36) was determined by immunoblotting with either anti-BRC36 or -FLAG antibodies. (**B**) 5 x 10^5 of MCF-7 (positive control), BRCC36 transfected- or vector control MCF-10 cells were plated in soft agar (6-well plates). After 3 weeks, images from 5 independent fields of each well were taken and colonies containing >30 cells were scored. The numbers of colonies shown are the means ± standard deviations (SD) of triplicate results from two independent experiments. (**C**) Representative images of colony formation in soft agar by MCF-7, BRCC36-transfected and vector control MCF-10F cells.

Characterization of New Polyclonal anti-BRCC36 Antibodies

We have developed two new polyclonal antibodies to different epitopes of BRCC36. As shown in **Figure 18A**, commercially available antibodies (Zymed) to the C-terminus and our antibodies derived against the N-terminus (a.a. 40-53) (NP_001018065, NCBI) detect both isoforms of BRCC36, while antibodies derived against amino acids encoded by sequences in exons 7 and 8, i.e., a.a. 176-189 (NP_001018065, NCBI), uniquely detect isoform 2 of BRCC36. Since the commercial BRCC36 antibodies do not work for immunoflorescence (IF)-based assays (**data not shown**), we are currently evaluating the sensitivity and specificity of our new antibodies for IF staining. As shown in **Figure 18B**, BRCC36 forms discrete nuclear foci in MCF-10F cells following exposure to ionizing radiation. These data are consistent with a recent study reporting the nuclear foci formation of exogenous HA tagged-BRCC36 in U2OS cells in response to DNA damage (Sobhian et al, 2007). These findings continue to indicate that BRCC36 plays an important role in DNA damage/repair pathways.

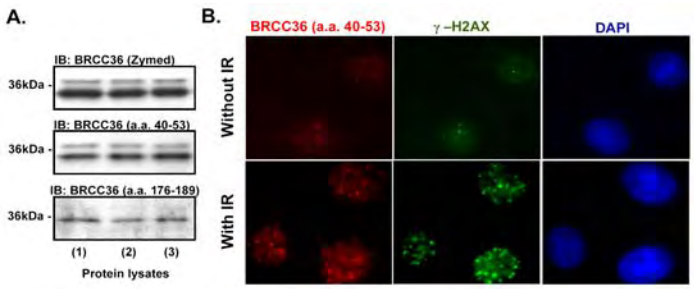

Figure 18. Characterization of New BRCC36 Antibodies. (**A**) Immunoblot analysis of BRCC36 in three independent lymphoblastoid cell lines using the Zymed polyclonal antibody (upper panel), antibodies derived against a.a. 40-53 (middle panel), and antibodies derived against a.a. 176-189 (lower panel). (**B**) IR-induced BRCC36 nuclear foci formation detected with antibodies derived against a.a. 40-53. MCF-10F cells were treated with 4-Gy IR and allowed to recover for 2 hours before performing immunoflorescence staining as described in our previous studies (Chen, et al, 2006). MCF-10F cells were also co-stained with γ-H2AX and DAPI for the nuclear foci positive control and nuclear visualization, respectively.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that BRCC36 is expressed at very low levels in normal breast epithelial cells and that it is highly expressed in breast tumors.

- Demonstrated that the aberrant expression of *BRCC36* is independent to *HER*-2/*neu* status in breast tumors.

- Demonstrated that BRCC36 interacted with BRCA1 in the MCF-7 cell line by co-immunoprecipitation.

- Demonstrated that cancer-causing truncations of BRCA1 abrogated the association of BRCC36 with BRCC.

- Reported that depletion of BRCC36 by the small interfering RNAs (siRNAs) resulted in increased sensitivity to ionizing radiation in three breast cancer lines.

- Reported that the abrogation of BRCC36 prevents the phosphorylation of BRCA1 and does not affect the levels of ATM, p53 and p21 in the breast cancer cells response to IR.

- Reported that the abrogation of BRCC36 disrupts the BRCA1 nuclear foci formation in the breast cancer cells following IR instead disrupts the BRCC complex integrity.

- Reported a frameshift *BRCC36* mutation (c.880insGGGGdup148) and a *CHEK2* c.1100delC) coexisted in the germline of a *BRCA1/2* mutation-negative breast cancer prone kindred.

- Demonstrated that a frameshift *BRCC36* mutation (c.880insGGGGdup148) results in a stabilized mutant transcript in lymphoblastoid cell lines (LCLs) by immortalizing lymphocytes collected from the proband.

- Identified an intron alteration (IVS2-29delT) in one primary breast tumor, which leads to in-frame 25 amino acids insertion.

- Evaluated eight male breast tumor specimens for *BRCC36* gene amplification, and no *BRCC36* gene amplification has been identified. However, one male breast tumor has been found to be hyperploids.

- Demonstrated that silencing of BRCC36 decreased the expression of MRE11, a subunit of the R/M/N complex.

- Established a functional GFP-based assay to assess specific double-strand break repair, and demonstrated that depletion of BRCC36 impairs homologous recombination repair (HRR).

- Demonstrated by protein structure analysis that BRCC36 contains a Jab1/MPN domain metalloenzyme (JAMM) motif, and the conserved BRCC36 residues form a catalytic site that binds a Zn atom. These findings from BRCC36 protein modeling suggest that BRCC36 may play a direct role in ubiquitination.

- Successfully delivering BRCC36 into MEC lines using an electroporation expression system, and performing two-dimensional gels using protein lysates from containing MCF-10A cell transfected with CMV-5a empty vector or MCF-10A cell transfected with BRCC36-Flag.

- Demonstrated that BRCC36 overexpression stimulates the colony formation in a human mammary epithelial cell line.

- Developed two new polyclonal antibodies to different epitopes of BRCC36, which are able to detect both isoforms of BRCC36 or uniquely detect isoform 2 of BRCC36.

REPORTABLE OUTCOMES

Abstracts

1. **Chen, X.**, Dong, Y., Hakimi, M.-A., Shiekhattar, R., and Godwin, A. K. Aberrant expression of BRCC36, a novel subunit of a BRCA1 E3 ubiquitin ligase complex, in sporadic breast cancer. In: Annual meeting of American Association of Cancer Research, (AB# 4235, poster), 2004.

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3. **Chen, X.**, Arciero, C. A., Wang, C., Broccoli, D., and Godwin, A. K. Inhibition of BRCC36, a novel subunit of a BRCA1 E3 ubiquitin ligase complex (BRCC), promotes ionizing radiation-induced apoptosis in breast cancer cells. In: Annual meeting of American Association of Cancer Research, (AB# 5701, poster), 2005.

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11. **Chen, X.,** Weaver, J., Bove, B.A., Vanderveer, L., Miron, A., Daly, M.B., Godwin. A.K. Allelic imbalance in BRCA1 and BRCA2 gene expression is associated with an increased breast cancer risk. In: Annual meeting of American Association of Cancer Research, (AB# 1926, poster presentation), 2008.

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Future Funding:

BC074303 (PI: Chen, X) 10/1/2008 - 9/30/2011
Army Role: PI
Antibody-Mediated BRCC36 Silencing: A Novel Approach for Targeted Breast Cancer Therapy
The major goals of this project are: 1) To establish siRNAs targeting BRCC36 specifically to breast cancer cells using a novel delivery system in which homing antibodies are coupled with an avid RNA-binding protein, i.e., protamine; and 2) To determine if abrogation of BRCC36 can sensitize breast tumor cells to DNA damage-based therapies using mouse xenograft models.

CONCLUSIONS

Through this DOD fellowship awards, we have evaluated the role of BRCC36 in the ATM-BRCA1 DNA repair pathway in breast cancer cells in response to IR. The key findings of this work lie in the following: 1) BRCC36 is overexpressed in vast majority of breast tumors; 2) Depletion of BRCC36 enhances IR-induced apoptosis of breast cancer cells; 3) BRCC36 abrogation inhibits the formation of BRCA1 nuclear foci following IR; 4) Silencing of BRCC36 prevents the IR-induced phosphorylation of BRCA1 while other IR-response proteins, such as ATM, p53 and p21, are

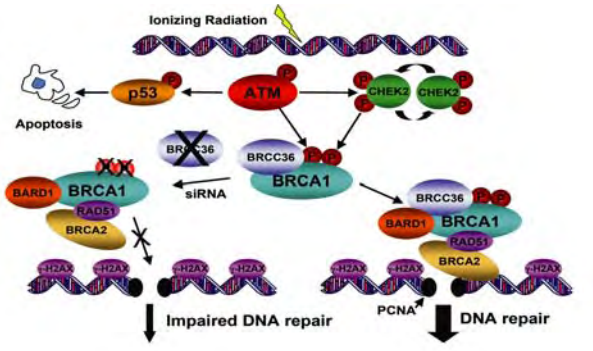

Figure 19. Potential Role of BRCC36 in the BRCA1-Associated DNA Repair Pathway in Response

unaffected; 5) Depletion of BRCC36 impairs homologous recombination repair (HRR); 6) Silencing of BRCC36 decreases the expression of MRE11, which mediates repair of DNA double strand break; and 7) *BRCC36* is mutated in the germline of a cancer-prone family and segregated with disease, suggesting to increase the risk of developing breast cancer. This *BRCC36* frameshift mutation results in expression of a mutant transcript in the immortalize lymphoblastoid cell lines.

In cellular response to the DNA damage caused by IR, ATM is activated by DNA damage and phosphorylates multiple factors, including BRCA1 and p53, which are involved in DNA repair, apoptosis and cell cycle arrest (Banin, et al., 1998; Canman, et al., 1998; Cortez, et al., 1999). As our results indicate, depletion of BRCC36 expression by RNAi blocks BRCA1 activation (i.e., phosphorylation and nuclear foci formation) in breast cancer cells following IR exposure. Because of the role of BRCA1 in DNA repair, we propose that an imbalance between the DNA repair/cell survival and DNA damage/cell apoptosis pathways exists in BRCC36-depleted cells following IR exposure. As a result, BRCC36 depletion appears to substantially sensitize breast cancer cells to IR-induced apoptosis (**Figure 19**). Overall, our studies define BRCC36 as a direct regulator of BRCA1 activation and nuclear foci formation in response to IR in a number of breast cancer cell lines. However, a conundrum exists in regards to the role of BRCC36 in the pathogenesis of breast cancer. We observed that BRCC36 is overexpressed in the vast majority of breast tumors, suggesting a potential oncogenic mechanism. However, we discovered a deleterious germline *BRCC36* mutation in a breast cancer prone-kindred that segregated with disease, suggesting a tumor suppressor activity. Therefore, BRCC36 may behave as either a tumor suppressor or an oncogene depending on the level of expression and the proteins it interacts with. This is consistent with our previous studies reporting the BRCC complex containing BRCA1-BARD1-BRCC45-BRCC36 has enhanced E3 ubiquitin ligase activity compared to that of BRCA1-BARD1 heterodimer (Dong, et al., 2003) and a recent study reporting DUB activity when presenting in BRCA1-BARD1-RAP80-BRCC36 (Sobhian, et al., 2007). These findings suggest that the balance between synthesis and turnover of certain polyubiquiquitinated structure by BRCA1-BARD1 E3 and BRCC36 DUB activities, respectively, could be dynamic and dependent on the level of BRCC36 and other protein partners (e.g., BRCC45 or RAP80) in the complexes. The data from these studies suggest that overexpression or inactivation of *BRCC36* via mutations disrupts the normal function of BRCA1 (i.e., leading to a BRCA1 null-like phenotype) and contribute to the development of breast cancer. Future efforts could be focused on defining if BRCC36 functions as a tumor suppressor or proto-oncogene by studying its roles in primary breast epithelial cell transformation. Future studies could also examine the role of Brcc36 in mammary tumorigenesis using mouse models.

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APPENDICES

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Research Article

BRCC36 Is Essential for Ionizing Radiation–Induced BRCA1 Phosphorylation and Nuclear Foci Formation

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Abstract

We have previously reported the identification and characterization of a novel BRCA1/2 interacting protein complex, BRCC (BRCA1/2-containing complex). BRCC36, one of the proteins in BRCC, directly interacts with BRCA1, and regulates the ubiquitin E3 ligase activity of BRCC. Importantly, *BRCC36* is aberrantly expressed in the vast majority of breast tumors, indicating a potential role in the pathogenesis of this disease. To further elucidate the functional consequence of abnormal *BRCC36* expression in breast cancer, we have done *in vivo* silencing studies using small interfering RNAs targeting *BRCC36* in breast cancer cell lines, i.e., MCF-7, ZR-75-1, and T47D. Knock-down of *BRCC36* alone does not affect cell growth, but when combined with ionizing radiation (IR) exposure, it leads to an increase in the percentage of cells undergoing apoptosis when compared with the small interfering RNA control group in breast cancer cells. Immunoblot analysis shows that inhibition of BRCC36 has no effect on the activation of ATM, expression of p21 and p53, or BRCA1-BARD1 interaction following IR exposure. Importantly, BRCC36 depletion disrupts IR-induced phosphorylation of BRCA1. Immunofluorescent staining of BRCA1 and γ-H2AX indicates that BRCC36 depletion prevents the formation of BRCA1 nuclear foci in response to DNA damage in breast cancer cells. These results show that down-regulation of BRCC36 expression impairs the DNA repair pathway activated in response to IR by inhibiting BRCA1 activation, thereby sensitizing breast cancer cells to IR-induced apoptosis. (Cancer Res 2006; 66(10): 5039-46)

Introduction

Breast cancer is the most common cancer affecting women, with a woman's lifetime risk of breast cancer at ~10% by the age of 80 years. In the U.S., it was estimated that in 2005, ~211,000 new cases of breast cancer were diagnosed, and >40,000 deaths resulted from this disease (1). Breast cancer is a genetically heterogeneous disease, and germ line mutations in *BRCA1* and *BRCA2* genes predispose women to early onset breast cancer and/or ovarian cancer (2, 3). Since their cloning and characterization in the mid-1990s (4, 5), BRCA1 and BRCA2 proteins have been implicated in many cellular processes, including DNA repair and cell cycle-checkpoint control (6–10). BRCA1 has also been reported to be involved in protein ubiquitylation and chromatin remodeling (11, 12). Despite the fact that *BRCA1* and *BRCA2* mutations contribute to hereditary breast/ovarian cancer predisposition, somatic mutations are rarely found in sporadic breast cancers (13–15). Nevertheless, evidence is accumulating that dysfunction of other genes, coding for proteins in similar or redundant pathways as BRCA1 and BRCA2, might be important in the pathogenesis of a significant fraction of nonfamilial breast cancers. This speculation comes from several lines of evidence, including both phenotypic analyses of breast and ovarian tumors, as well as mechanistic studies of BRCA1- and BRCA2-associated pathways (16–18).

We have previously reported a novel multiprotein complex, termed BRCC, containing seven polypeptides including BRCA1, BRCA2, BARD1, and RAD51 (19). BRCC is an ubiquitin E3 ligase complex exhibiting an E2-dependent ubiquitination of the tumor suppressor p53. In this multiprotein complex, one of these proteins, referred to as BRCC36, has been found to be associated with BRCA1 and BRCA2, and has been shown to play an important role in the regulation of the ubiquitin E3 ligase activity of BRCC. The *BRCC36* gene is located at the *Xq28* locus, a chromosomal break point in patients with prolymphocytic T cell leukemia (20). BRCC36 displays sequence homology with the human Poh1/Pad1 subunit of the 26S proteasome and with subunit 5 (Jab1) of the COP9 signalosome (19). We have shown that cancer-associated mutations in BRCA1 abrogated the association of BRCC36 with BRCC and BRCA1 (19). Furthermore, reconstitution of a recombinant four-subunit BRCC complex containing BRCA1/BARD1/BRCC45/BRCC36 reveals an enhanced E3 ligase activity compared with that of BRCA1/BARD1 heterodimer (19). In addition, we have reported aberrant expression of *BRCC36* in the majority of breast cancer cell lines and invasive ductal carcinomas (19). The mechanism and consequences of abnormal *BRCC36* expression in breast cancer are presently unknown.

Previous studies have shown that BRCA1 is activated via the ATM/CHEK2 (CHK2) signaling pathway following the exposure of cells to DNA-damaging agents such as ionizing radiation (IR; refs. 21, 22). Following IR, BRCA1 is phosphorylated and forms discrete nuclear foci (dots) in response to DNA damage (23). Because BRCC36 directly interacts with BRCA1, we investigated the effects of knocking down BRCC36 expression, using small interfering RNAs (siRNA) on the growth and apoptosis of breast cancer cells. We further determined the role of BRCC36 in the BRCA1-associated DNA repair pathway activation following DNA damage. Here, our studies show that BRCC36 is a direct regulator of BRCA1 activation in response to IR.

Materials and Methods

Cell culture, siRNA transfection, and IR. Nontumorigenic epithelial cell lines, MCF-10F and 12A were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM/F12 with reduced Ca^{2+} (0.04 mmol/L final), 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 0.01 mg/mL insulin, 500 ng/mL hydrocortisone, and 5%

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doi:10.1158/0008-5472.CAN-05-4194

Chelex-treated horse serum. The human breast cancer cell lines, MCF-7, T47D, and ZR-75-1, were also obtained from the American Type Culture Collection. MCF-7 cells were maintained in DMEM medium, supplemented with 10% fetal bovine serum, penicillin, and streptomycin. T47D and ZR-75-1 cells were maintained in RPMI 1640, supplemented with 10% fetal bovine serum, penicillin, and streptomycin. 293-BARD1 cells were generously provided by Dr. R. Shiekhattar (Wistar Institute, Philadelphia, PA) and were maintained in MEM (Eagle) with 10% heat-inactivated horse serum.

For the *BRCC36* depletion studies, breast cancer cells were plated at a density of 5×10^3 cells/cm^2. After reaching 30% to 40% confluence, cells were transfected with siRNA using OligofectAMINE and OPTI-MEM I reduced serum medium (Invitrogen/Life Technologies, Inc., Carlsbad, CA) according to the manufacturer's protocol. The siRNA sequences targeting *BRCC36* corresponded to the coding region 253 to 273 bp (5′-AAGAG-GAAGGACCGAGTAGAA-3′) relative to the start codon. The corresponding siRNA duplexes with the following sense and antisense sequences were used: 5′-GAGGAAGGACCGAGUAGAAdTdT (sense) and 5′-UUCUACUCG-GUCCUUCCUCdTdT (antisense). This siRNA has been used in a previous study (19), as well as another siRNA targeting BRCC36 (corresponding to the coding region 120-138 bp). Both resulted in similar levels of transcript depletion. Green fluorescent protein siRNA was used as the negative control. All of the siRNA duplexes were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using 2′-ACE protection chemistry. Twenty-four hours after the initial transfection, cells were subcultured and replated at 5×10^3 cells/cm^2. The cells were then retransfected under similar conditions 24 hours after replating. Seventy-two hours after the first transfection, the cells were irradiated using a Cesium 137 irradiator (model 81-14R). The cells received 4 Gy total IR (1.132 Gy/min for 3.53 minutes) for a targeted 50% induction of apoptosis (24). Cells were then grown for an additional 72 hours prior to harvesting and further analyses.

RNA isolation, reverse transcription, and quantitative PCR. Total cellular RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the protocols provided by the manufacturer. Total RNA (2 μg) was used as a template to be reverse-transcribed in a 20 μL reaction containing 5 μmol/L random hexamers, 500 μmol/L of deoxynucleoside triphosphate mix, 1× reverse transcriptase buffer, 5 mmol/L $MgCl_2$, 1.5 units of RNase inhibitor, and 7.5 units of MuLV reverse transcriptase. All reagents were obtained from Applied Biosystems (Branchburg, NJ). The reaction conditions were as follows: 10 minutes at 25°C, 1 hour at 42°C, and 5 minutes at 94°C. The cDNA mixture (0.625 μL) was used in a real-time PCR reaction (25 μL total volume) done with ABI 7900HT (Applied Biosystems) following protocols recommended by the manufacturer. Optimal conditions were defined as: step 1, 95°C for 10 minutes; step 2, 95°C for 15 seconds, 60°C for 60 seconds with Optics, repeated for 40 cycles. The relative mRNA expressions of *BRCC36* were adjusted with β-actin (ACTB). The primer and probe sets used for real-time PCR were as follows: *BRCC36*, forward primer, 5′-AATTTCTCCAGAGCAGCTGTCTG; reverse primer, 5′-CATGGCTTGTGTGCGAACAT; TaqMan probe, (FAM) 5′-AACTGACAGGCCGCC-CCATGAG-(BHQ1); β-actin, forward primer, 5′-GCCAGGTCATCACCATTGG; reverse primer, 5′-GCGTACAGGTCTTTGCG-GAT; TaqMan probe, (Cal red) 5′-CGGTTCCGCTGCCCTGAGGC-(BHQ2).

Coimmunoprecipitation. 293 cells that stably express FLAG-BARD1 (19) at 70% to 80% confluence were washed twice with ice-cold D-PBS before scraping on ice with lysis buffer [50 mmol/L Tris-HCl (pH 7.4), with 150 mmol/L NaCl, 1 mmol/L EDTA, and 1% Triton X-100 and one tablet of protease inhibitor mixture per 40 mL of lysis buffer (Roche Molecular Biochemicals, Indianapolis, IN)]. Cellular debris was removed by centrifugation (14,000 × *g* for 15 minutes at 4°C), and protein concentrations were determined using the Bio-Rad detergent-compatible protein assay reagent. Cell lysate (1.5 mg) was added to the anti-FLAG M2-agarose affinity gel (Sigma, St. Louis, MO). All samples were placed on a roller shaker overnight at 4°C. After centrifugation, the supernatants were removed and the gel beads were washed thrice with 0.5 mL of wash buffer [50 mmol/L Tris-HCl (pH 7.4), with 150 mmol/L NaCl]. The beads were washed an additional four times with the wash buffer, resuspended in 20 μL of 2× SDS sample buffer before boiling for 5 minutes. Fifteen microliters of immunoprecipitate were separated by SDS-PAGE electrophoresis on 4% to 20% and 5% linear gradient Tris-HCl ready gels (Bio-Rad, Richmond, CA).

Western blot and antibodies. Cells were homogenized in lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2.5 mmol/L Na-pyrophosphate, 1 mmol/L Na-β-glycerophosphate, 20 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, one tablet of protease inhibitor mixture (Roche Molecular Biochemicals)]. Cellular debris was removed by centrifugation as above. Protein concentrations were determined with Bio-Rad detergent-compatible protein assay (Bio-Rad). For BRCA1 and pATM, cells were lysed directly in Laemmli sample buffer (Bio-Rad). Proteins were resolved on 5% (BRCA1 and pATM) or 4% to 20% linear gradient (β-actin, p53, p21, and RAD51) SDS-PAGE ready gels at 120 V for 1.5 to 3 hours with 1× SDS running buffer. SDS-PAGE gels were transferred onto an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). Primary antibodies used for Western analysis were mouse anti-BRCA1 (1:100; EMD Biosciences, San Diego, CA), mouse anti-phosphorylated ATM-S1981 (1:500; Rockland, Gilbertsville, PA), mouse anti-p53 (1:1,000, Cell Signaling, Beverly, MA), mouse anti-p21^{WAF} (1:100, EMD Biosciences), mouse anti-RAD51 (1:500; Upstate Biotechnology, Lake Placid, NY), mouse anti-FLAG M2 (1:1,000; Sigma), and mouse anti-β-actin (1:5,000; Sigma). Secondary antibodies were mouse and rabbit IgG, horseradish peroxidase–linked (1:10,000; Amersham, Piscataway, NJ). Perkin-Elmer Life Sciences renaissance enhanced luminol reagents (Boston, MA) were used as substrates for detection. To reprobe immunoblot membranes, Restore Western blot stripping buffer (Pierce, Rockford, IL) was used to strip the membrane.

Apoptosis assay. Breast cancer cells were collected by trypsinization and pelleted by centrifuging for 5 minutes at 800 × *g* at 4°C. After washing with 1 mL of ice-cold 1× Nexin buffer (Guava Technologies, Hayward, CA), the cells were resuspended in 100 μL of the same buffer. After labeling with Annexin V and 7-amino actinomycin D, the proportion of apoptotic cells was determined using a Guava personal cytometer (Guava Technologies) according to the manufacturer's instruction. Cell apoptosis was also analyzed using a terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) assay. In brief, breast cancer cells were grown in four-well chamber slides (Nalge Nunc International, Rochester, NY). After fixing with 4% paraformaldehyde in PBS and permeabilizing with 0.1% Triton X-100 in 0.1% sodium citrate solution, apoptotic cells were detected using an *in situ* cell death detection kit (Roche, Germany) according to the manufacturer's instructions. At least 1,200 cells from eight independent fields were counted to evaluate the percentage of apoptotic cells.

Immunofluorescence and antibodies. MCF-7, ZR-75-1, and T47D cells were grown in four-well chamber slides (Nalge Nunc International) and processed for immunofluorescent analysis as described previously (25). For nuclear foci formation and colocalization of the BRCA1 and γ-H2AX, cells were preextracted in protein extraction solution (20 mmol/L HEPES, 50 mmol/L NaCl, 3 mmol/L $MgCl_2$, 300 mmol/L sucrose, and 0.5% Triton X-100), fixed in 3.7% formaldehyde (Fisher, Pittsburgh, PA) in PBS for 10 minutes and permeabilized in 0.5% NP40 in PBS prior to incubation with the following antibodies: rabbit anti-BRCA1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-γ-H2AX (1:200; Upstate Biotechnology). Primary antibodies were detected with tetramethyl rhodamine isothiocyanate–conjugated donkey anti-rabbit IgG and FITC-conjugated donkey anti-mouse IgG (1:100; Jackson ImmunoResearch, West Grove, PA). DNA was counterstained with 0.1 μg/mL of 4′,6′-diamidino-2-phenylindole (Sigma) and mounted in embedding medium (0.1% *p*-phenylene diamine in 90% glycerol, 1× PBS). Microscopic analysis was carried out using the Eclipse TE2000 (Nikon, Melville, NY) and images captured using a Cascade 650 monochrome camera (PhotoMetrics, Huntington Beach, CA). A series of 0.5-μm sections were collected for seven fields of each treatment group. Image acquisition from a Cascade 650 monochrome camera (PhotoMetrics) was controlled by MetaVue (v6.2r6, Universal Imaging/Molecular Devices, Downingtown, PA). An automated Ludl MAC2000 *x-y* stage and *z*-axis motor were also controlled using the MetaVue software (v6.2r6).

Image analysis. Quantification of BRCA1 and γ-H2AX nuclear foci formation was done with Metamorph software (v6.1; Universal Imaging/

Molecular Devices). In brief, a series of *Z*-sections for each channel was reassembled using the "maximum" type option within the "3-D reconstruction" function. Nuclei were defined in the 4′,6′-diamidino-2-phenylindole channel using the functions of "threshold for light objects" and "create regions from objects." Adjacent nuclei were separated into independent regions using the "cut-drawing" tool. Regions were then transposed onto the reassembled image for each digital channel, after background was removed using the "flatten background" function, and positive signals were identified by manual thresholding (high, 2,836; low, 1,758). For each nucleus, the number of the BRCA1 or γ-H2AX foci was calculated using the "foci measure" function. Approximately 70 cells of each treatment group from seven independent fields were analyzed to evaluate the number of BRCA1 foci.

Statistical analysis. Student's *t* test was employed using SAS software 8.0 (SAS Institute, Cary, NC). $P < 0.05$ was considered significant and results were presented as the mean ± SD.

Results

Inhibition of *BRCC36* gene expression by siRNA. To further elucidate the functional consequence of *BRCC36* aberrant expression in the pathogenesis of breast cancer, we did *in vivo* silencing studies targeting *BRCC36* in MCF-7, T47D, and ZR-75-1 breast cancer cell lines, which constitutively expresses high levels of *BRCC36* transcript relative to nontumorigenic breast epithelial cells, i.e., MCF-10F and MCF12A (Fig. 1*A*). Because antibodies specific to BRCC36 protein are not available, quantitative PCR was used to establish constitutive and attenuated *BRCC36* mRNA levels. We used siRNA targeting *BRCC36* or green fluorescent protein (negative control) to assess the response of BRCC36 depletion in breast cancer cells. Various siRNAs to BRCC36 were previously evaluated (19) and shown to be effective alone or in combination; however, for the purpose of these studies, *BRCC36*-siRNA1 was used. Treatment with this siRNA resulted in a >50% to 80% decrease in *BRCC36* mRNA levels in comparison to mock-treated or siRNA control–transfected cells ($P < 0.05$) by 72 hours. The greatest level of suppression was observed in MCF-7 cells (Fig. 1*B*).

Abrogation of *BRCC36*-enhanced IR-induced breast cancer cell apoptosis. Following depletion of *BRCC36* via siRNA, MCF-7, ZR-75-1, or T47D were treated with and without IR (4 Gy). The cells were cultured for an additional 72 hours prior to harvesting and were examined for DNA damage–induced cell apoptosis via Annexin V and 7-amino actinomycin D staining. No significant difference in the fraction of cells undergoing apoptosis in mock-treated, siRNA control-transfected, or siRNA-*BRCC36*–transfected cells was observed in the absence of IR, indicating that depletion of BRCC36 alone is not lethal (Fig. 2*A*; data not shown). However, when combined with *BRCC36* knock-down, IR exposure led to a significant increase in the percentage of MCF-7 cells that undergo apoptosis (45.9 ± 4.3%) when compared with the siRNA control group (34.9 ± 1.9%, $P < 0.05$; Fig. 2*A*; Table 1). Consistent with these results, the overall cell viability was substantially lower in siRNA-*BRCC36*–treated cells following IR as compared with control cells (50.9 ± 5.8% versus 58.4 ± 5.7%; Table 1). Similar results were observed in T47D cells treated with siRNA-BRCC36 and IR versus controls (42.2 ± 4.5% versus 23.3 ± 1.9%, $P < 0.05$; Fig. 2*A*; Table 1). Although the trend was evident for ZR-75-1 cells, the fraction of cells undergoing apoptosis following depletion of *BRCC36* and IR were not statistically significantly different (data not shown). Induction of apoptosis was confirmed using a TUNEL assay and MCF-7 cells (Fig. 2*B*). The combination of *BRCC36* siRNA abrogation and IR exposure again resulted in a significant increase in the fraction of cells undergoing apoptosis when compared with the siRNA control–treated cells (40.9 ± 2.7% versus 24.9 ± 3.3%, $P < 0.05$; Fig. 2*C*).

Inhibition of BRCC36 disrupted BRCA1 phosphorylation in breast cancer cells exposed to IR. Previous studies have indicated that the BRCA1 protein is phosphorylated in response to DNA-damaging agents (23). Because BRCC36 directly interacts with BRCA1 (19), we examined the effect of BRCC36 depletion on BRCA1-associated DNA repair/damage pathways. MCF-7 cells were treated with siRNA targeting *BRCC36*, and then exposed to 4 Gy of IR to induce DNA damage. MCF-7 cells were harvested 2 hours after IR. Western blot analysis was carried out to examine the expression and modification of BRCA1, p21, p53, and ATM. Western analysis clearly shows that DNA damage induced by IR resulted in increased expression of p21, stabilization of p53, and phosphorylation of BRCA1 and ATM (S1981) as expected (Fig. 3). This same pattern was observed in siRNA control–treated cells. Importantly, the reduction of BRCC36 blocked IR-induced phosphorylation of BRCA1. In comparison, BRCC36 knock-down had no effect on IR-induced expression of p21, stabilization of p53, and phosphorylation of ATM.

Effects of inhibition of BRCC36 on integrity of BRCA1-BARD1 heterodimer. To determine if depletion of BRCC36 affects the integrity of the BRCA1-BARD1 heterodimer, we targeted *BRCC36* mRNA in a 293-derived cell line expressing FLAG-BARD1

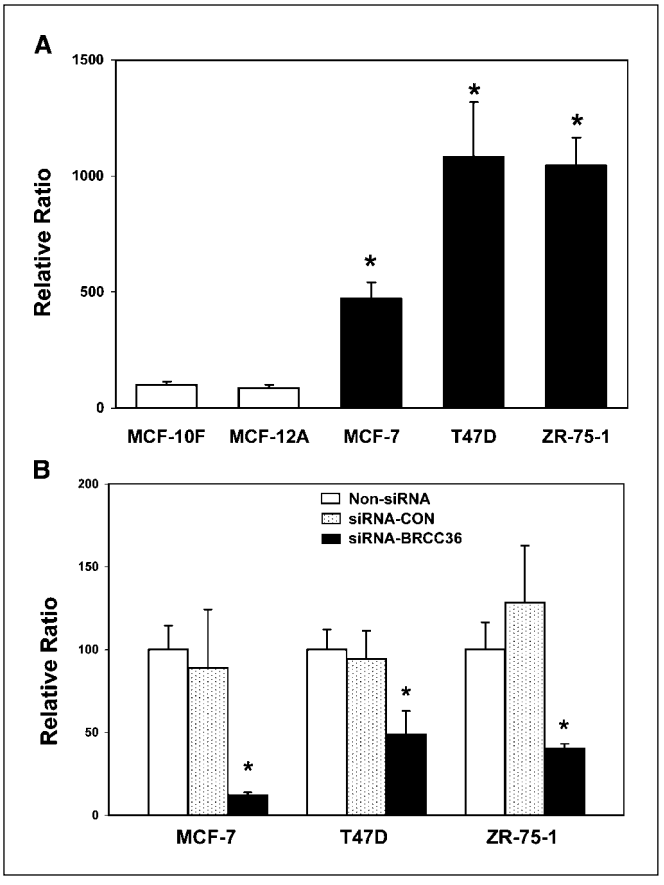

Figure 1. Abrogation of *BRCC36* expression by siRNA treatment. Quantitative reverse transcription-PCR analysis was done to examine the gene expression of *BRCC36*. The relative level of *BRCC36* expression was adjusted with β-actin (*ACTB*). *A*, *BRCC36* expression in nontumorigenic (*MCF-10F* and *MCF12A*) and tumorigenic (*MCF-7, ZR-75-1*, and *T47D*) breast epithelial cell lines. *B*, *BRCC36* expression in breast cancer cell lines after siRNA treatment ($P < 0.05$).

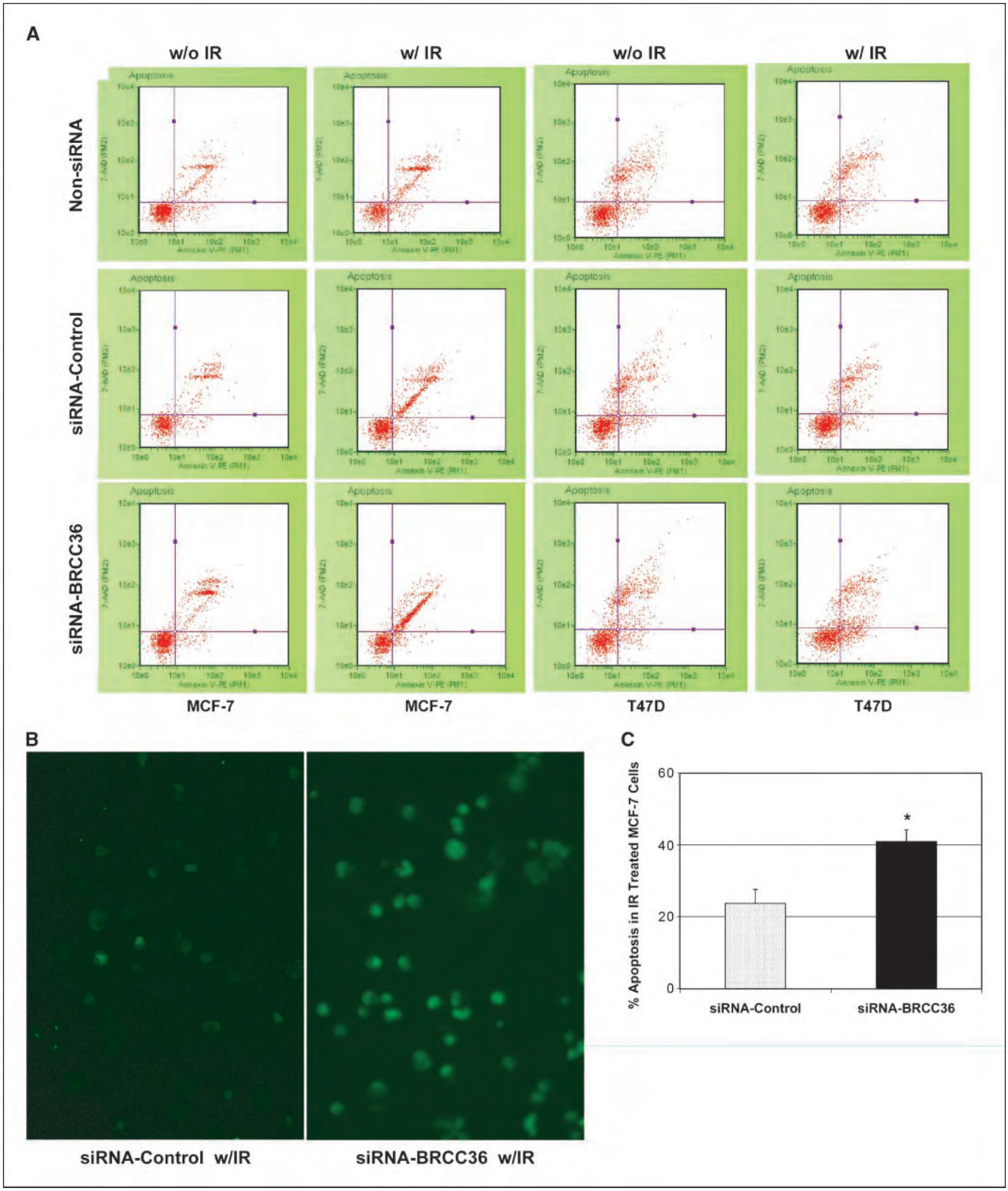

Figure 2. Apoptosis analysis in breast cells exposed to IR. *A,* MCF-7 and T47D cells were mock treated (*non-siRNA*) or were transfected with siRNA-control or siRNA-*BRCC36* prior to IR exposure. The proportion of apoptotic cells was measured following Annexin V and 7-amino actinomycin D staining using a Guava personal cytometer. All studies were done in triplicate. *B,* TUNEL labeling was done to detect apoptotic MCF-7 cells (*light green*) following exposure to IR. *C,* data analysis of the TUNEL assay. At least 1,200 cells of each treatment group from eight independent fields were counted to evaluate the percentage of apoptotic cells.

Table 1. Nexin assay in breast cancer cells exposed to IR

	MCF-7		T47D	
	Viable cells (%)	Apoptotic cells (%)	Viable cells (%)	Apoptotic cells (%)
Non-siRNA	57.3 ± 7.2	34.4 ± 4.5	68.5 ± 0.3	26.5 ± 0.4
siRNA-Control	58.4 ± 5.7	34.9 ± 1.9	70.5 ± 2.3	23.3 ± 1.9
siRNA-BRCC36	50.9 ± 5.8	45.9 ± 4.3*	52.7 ± 2.4	42.2 ± 4.5*

*siRNA-BRCC36 versus siRNA-Control (Student's t test, $P < 0.05$).

(19). Immunoprecipitation was done using anti-FLAG on lysates prepared from cells transfected with the BRCC36 or control siRNAs. BRCA1 and BARD1 were examined with SDS-PAGE and Western blot. Reduction of BRCC36 did not seem to alter the BRCA1-BARD1 interaction in either untreated or IR-treated cells (Fig. 4).

Inhibition of BRCC36 disrupts BRCA1 nuclear foci formation in breast cancer cells exposed to IR. It is well characterized that BRCA1 localizes to discrete nuclear foci (dots) during S phase or in response to DNA damage. In our previous report, we showed that BRCC36 directly interacts with BRCA1 at the region encompassing amino acids 502 to 1,054 (19). This region falls within the BRCA1 DNA-binding domain (amino acids 452-1079). Because the DNA-binding domain has been shown to contribute to the BRCA1 relocalization after DNA damage (23, 26), we sought to evaluate the role of BRCC36 in the formation of BRCA1 nuclear foci in response to DNA damage. MCF-7, T47D, and ZR-75-1 cells, mock-treated or transfected with siRNA-control or siRNA-*BRCC36*, were exposed to IR (4 Gy), and were evaluated for BRCA1 and γ-H2AX subcellular location. Recent studies have shown that the three breast cancer cell lines used in this study possess wild-type *BRCA1* (27). As shown in Fig. 5, BRCC36 deficiency inhibits BRCA1 focus formation as compared with mock-treated and siRNA control–transfected cells. Importantly, γ-H2AX response to IR was unaffected in the cells transfected with BRCC36 siRNA (Fig. 5*A-C*). Quantification of BRCA1 nuclear foci showed that siRNA-BRCC36 transfection in MCF-7 cells resulted in 63% and 52% decrease compared with siRNA-control cells at 2 and 4 hours post-IR, respectively ($P < 0.05$; Fig. 5*D* and *E*). Similar results were observed in T47D (49% and 36%) and ZR-75-1 (59% and 71%) cells (Fig. 5*D* and *E*). Collectively, these results show that down-regulation of BRCC36 expression impairs the DNA repair pathway activated in response to IR by inhibiting BRCA1 activation.

Discussion

In this study, we have evaluated the role of BRCC36 in the ATM/BRCA1 DNA repair pathway in breast cancer cells in response to IR. The key findings of this work lie in the following: first, we have shown that the depletion of *BRCC36* mRNA enhances IR-induced apoptosis of breast cancer cells (Fig. 2). Second, the reduction of BRCC36 prevents the IR-induced activation of BRCA1 whereas other IR response proteins, such as ATM, p53 and p21, are unaffected (Fig. 3). Third, BRCC36 abrogation inhibits the

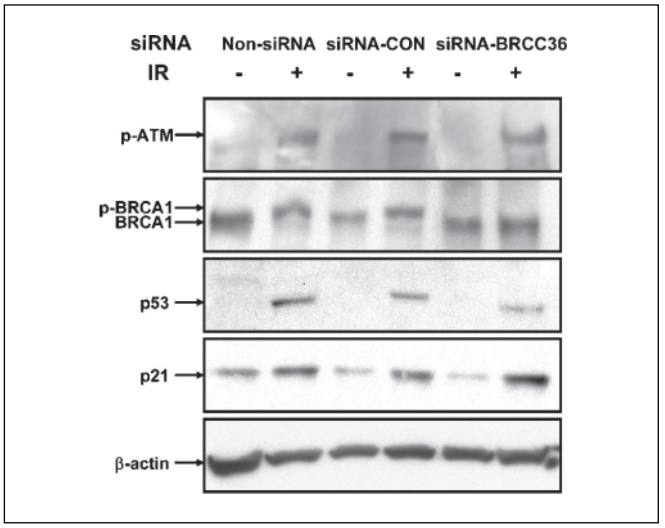

Figure 3. Activation of BRCA1 in response to IR treatment. MCF-7, MCF-7/siRNA control and MCF-7/siRNA-*BRCC36* cells were treated with or without IR (4 Gy), and cells were evaluated 2 hours after radiation exposure. BRCA1 protein was evaluated by immunoblotting with anti-BRCA1 antibody and shifts in the mobility of the protein bands indicated phosphorylated and unphosphorylated protein. The protein levels of phosphorylated ATM, p53, and p21 were determined by immunoblotting with anti-p-ATM, anti-p53, and anti-p21 antibodies, respectively. Protein loading levels were evaluated by immunoblotting with anti-β-actin antibody.

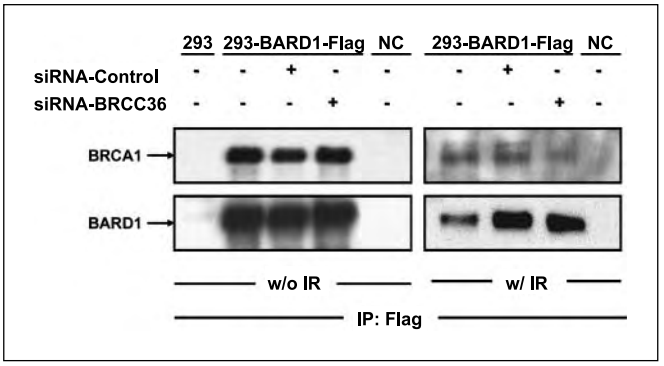

Figure 4. Effects of inhibition of BRCC36 on the integrity of BRCA1-BARD1 heterodimer. 293-BARD1-FLAG cells were transfected with either siRNA-*GFP* (*siRNA Control*) or siRNA-*BRCC36*. Transfected cells were then treated with 4 Gy IR and were incubated for 2 hours before harvesting. 293 cell lysate (1.5 mg; control or BRCC36-siRNA transfected) was incubated with ANTI-FLAG M2-agarose affinity gel. Immunoprecipitates were separated by SDS-PAGE electrophoresis. The protein levels of BRCA1 and BARD1 were determined by immunoblotting with anti-BRCA1 and anti-BARD1 antibody, respectively (*NC,* negative control).

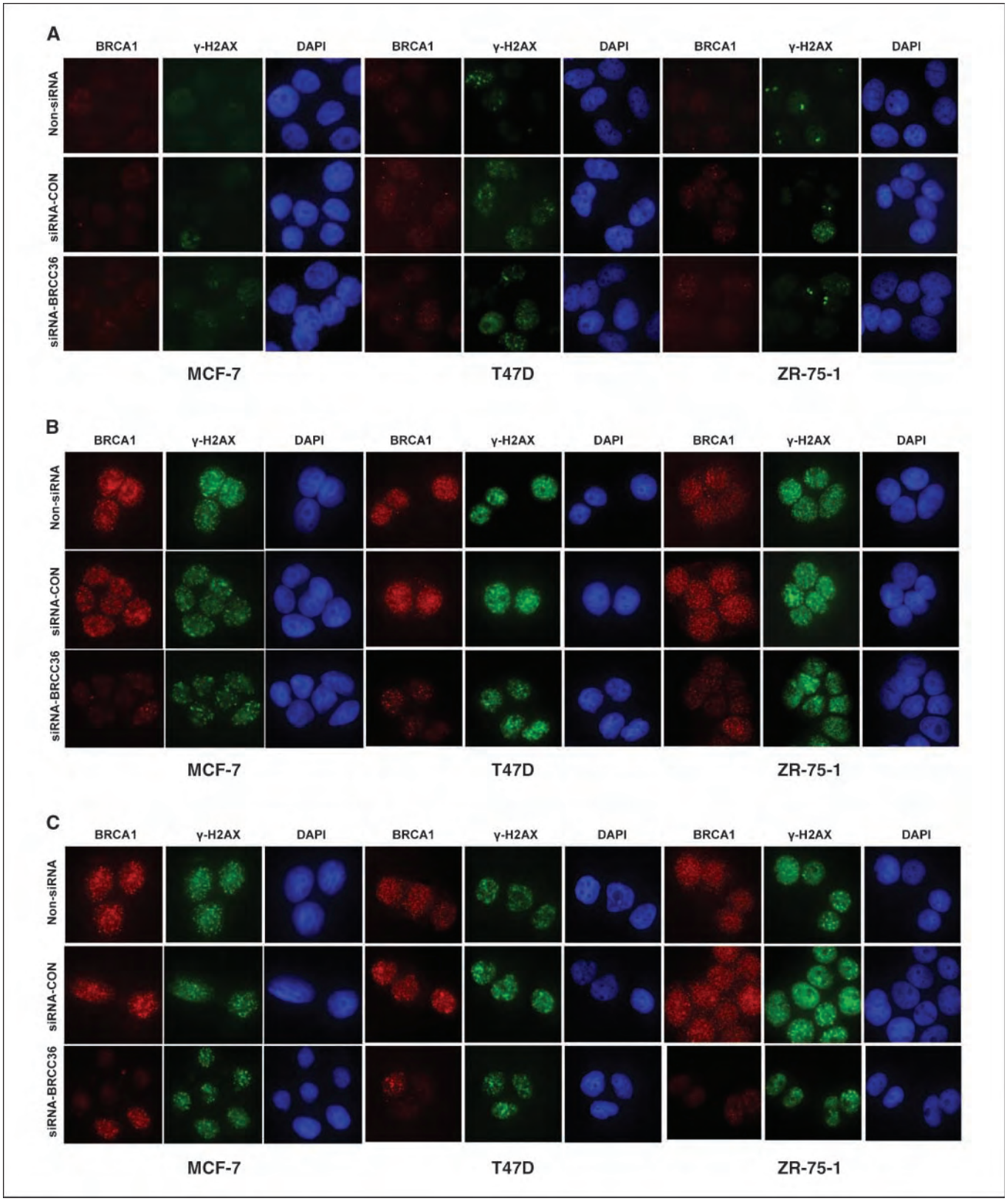

Figure 5. BRCA1 nuclear foci formation in breast cancer cells following IR exposure. MCF-7, ZR-75-1, and T47D cells were mock-treated (*non-siRNA*) or transfected with siRNA-CON or siRNA-BRCC36. Transfected cells were then treated with 4 Gy IR and were incubated for 2 or 4 additional hours. After pre-extraction and fixation, transfection cells then were immunostained for BRCA1 and γ-H2AX. Microscopic analysis was carried out using the Nikon Eclipse TE2000 and a Cascade 650 monochrome camera. Quantification of BRCA1 nuclear foci formation was done with Metamorph software (v6.1.). *A,* BRCA1 and γ-H2AX nuclear foci formation without IR exposure. *B,* BRCA1 and γ-H2AX nuclear foci formation at 2 hours post-IR exposure. *C,* BRCA1 and γ-H2AX nuclear foci formation at 4 hours post-IR exposure.

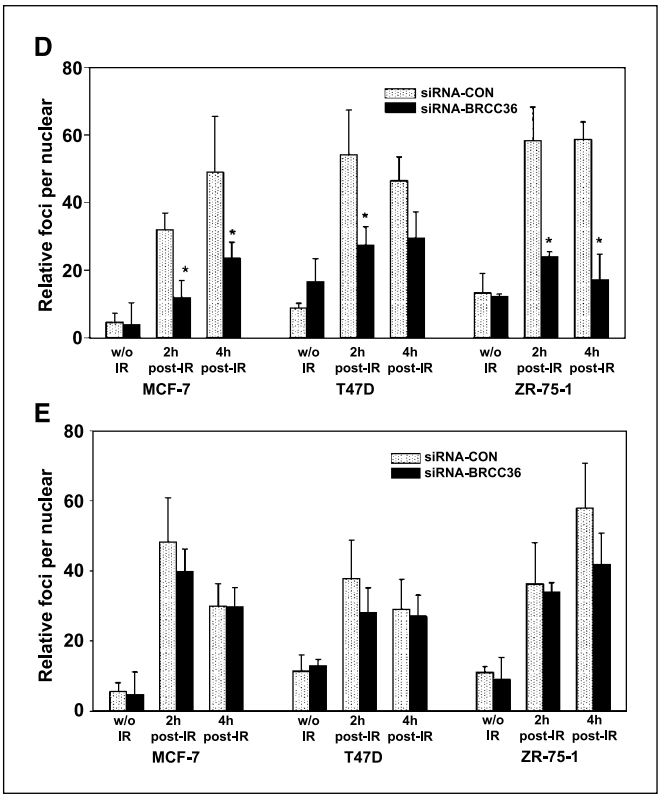

Figure 5 *Continued.* Quantification of BRCA1 (*D*) and γ-H2AX (*E*) nuclear foci formation without IR or at 2 and 4 hours post-IR exposure. Approximately 70 cells in each treatment group from seven independent fields were analyzed to evaluate the number of BRCA1 or γ-H2AX nuclear foci.

formation of BRCA1 nuclear foci following IR, without preventing the interaction of BRCA1 with its well-characterized binding partner, BARD1 (Figs. 4 and 5; data not shown).

The damage caused by IR activates various DNA repair pathways, including the ATM/ATR/CHEK2 pathways (21, 28). The central component of these DNA repair pathways is ATM kinase (29). ATM is activated by DNA damage and phosphorylates multiple factors, including BRCA1 and p53, which are involved in DNA repair, apoptosis and cell cycle arrest (21, 30, 31). As our results indicate, depletion of BRCC36 expression by siRNAi blocks BRCA1 activation, i.e., phosphorylation and nuclear foci formation in breast cancer cells following IR exposure, but has no direct effect on IR-induced apoptosis. Because of the role of BRCA1 in DNA repair, we propose that disrupting BRCA1 activation by BRCC36 depletion creates an imbalance between the DNA repair/cell survival and DNA damage/cell apoptosis pathways in cells following IR exposure (Fig. 6). As a result, BRCC36 depletion seems to substantially sensitize breast cancer cells to IR-induced apoptosis. However, it should be noted that these studies were done in a limited number of breast cancer cell lines, the caveat being that the DNA damage response may be altered in any or all cancer cell lines.

BRCA1 has been examined for a possible role in the development of radioresistant breast tumors. In fact, researchers have reported that BRCA1-deficient breast cancer cells have an increased sensitivity to IR (32). More recent studies have focused on the genes that code for proteins with equivalent/complementary functions to BRCA1 or function in the same pathway as BRCA1. A number of studies (33–37) have reported that manipulation of BRCA1-associated proteins affects cellular resistance or sensitivity to IR. The abnormal change (loss or gain) of any component in these BRCA1-related protein complexes may lead to their functional defects, which would result in a "BRCA1 null" phenotype. This may begin to explain why BRCA1 itself is rarely mutated and only occasionally (~10%) epigenetically down-regulated in sporadic diseases (18, 38). Therefore, BRCA1-associated proteins, including BRCC proteins, may serve as potential targets for the treatment of breast cancer, including radiation therapy.

In a previous report, we have shown that BRCC36 directly interacts with amino acids 502 to 1,054 of BRCA1 (19). In our current study, we have found that IR induced-BRCA1 nuclear foci formation is disrupted in BRCC36-depleted breast cancer cells (Fig. 5). The mechanism by which BRCC36 interferes with IR induced-BRCA1 localization is not clear. Previous studies have shown that BRCA1 consists of a DNA-binding domain region encompassing amino acids 452 to 1,079, and this BRCA1 DNA-binding domain contributes to the DNA repair–related functions of BRCA1, including the BRCA1 relocalization after DNA damage

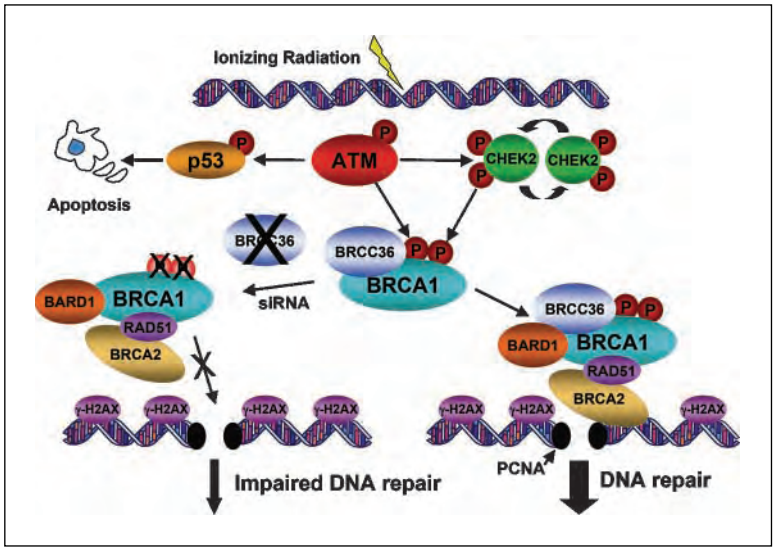

Figure 6. Model illustrating the potential role of BRCC36 in the BRCA1-associated DNA repair pathway in response to IR. BRCA1, p53, and CHEK2 are phosphorylated by ATM following DNA damage by IR; BRCA1 and p53 are involved in DNA repair and apoptosis, respectively. This activation leads to recruitment of many proteins to the site of the DNA damage, including BARD1, RAD51, BRCA2, and presumably BRCC36. Depletion of BRCC36 via siRNAs prevents the phosphorylation of BRCA1 and disrupts BRCA1 nuclear foci formation following IR, whereas γ-H2AX remains associated with regions of DNA damage. Due to the role of BRCA1 in DNA repair, the balance between the DNA repair/cell survival and DNA damage/cell apoptosis is disrupted and depletion of BRCC36 therefore sensitizes breast cancer cells to IR-induced apoptosis.

(23). The function of BRCA1 DNA-binding domain has been reported to be partially mediated through a protein complex, termed as BRCA1-associated surveillance complex (BASC; ref. 26). Interestingly, the location of BRCA1 DNA-binding domain coincides with the region that BRCC36 binds to, i.e., amino acids 452 to 1,079 versus amino acids 502 to 1,054 of BRCA1, respectively. In this study, we have found that depletion of BRCC36 by siRNA knock-down prevents the phosphorylation of BRCA1 following IR (Fig. 3). Previous studies have shown that BRCA1 is bound and phosphorylated by the ATM kinase and the G_2-M control kinase (CHEK2) after IR (21, 39, 40). Coincidentally, a host of studies have suggested that ATM and CHEK2 also bind to this central region of BRCA1 [reviewed by Narod and Foulkes (38)]. These findings may provide insight as to why depletion of BRCC36 in our studies inhibits BRCA1 activation, e.g., BRCC36 could help recruit BRCA1 to ATM and CHEK2 or stabilize their interactions following activation of the DNA damage response pathway. Our future studies are geared towards determining if BRCC36 remains associated with activated BRCA1 or whether BRCC36 must be displaced prior to phosphorylation by ATM and CHEK2. We have begun to explore these questions and have found that BARD1 and RAD51 remain associated with BRCA1 following BRCC36 depletion (Fig. 4; data not shown). However, we have yet to determine if BRCC36 depletion affects the interaction between BRCA1 and ATM and/or CHEK2 (data not shown).

Overall, our studies define BRCC36 as a direct regulator of BRCA1 activation and nuclear foci formation in response to IR in a number of breast cancer cell lines. Our results suggest that down-regulation of BRCC36 expression impairs the DNA repair pathway activated in response to IR and seems to sensitize breast cancer cells to IR-induced apoptosis. Therefore, it is intriguing to speculate that targeting BRCC36 may aid in the treatment of radiation-resistant breast tumors.

Acknowledgments

Received 11/23/2005; revised 3/7/2006; accepted 3/10/2006.

Grant support: AACR-Anna Barker Award for basic research (X. Chen), Cheryl Herman and the Eileen Stein-Jacoby Fund, and grants from the Department of Defense, DAMD17-03-1-0707 (A.K. Godwin), and W81XWH-04-1-0573 (X. Chen). This project was also funded, in part, under a grant from the Pennsylvania Department of Health. The Department specifically disclaims responsibility for any analyses, interpretations, or conclusions.

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We thank Dr. A. Knudson for his thoughtful comments, Dr. R. Shiekhattar for generously providing the 293-BARD1 cell line, Dr. J. Johnson for the help in Metamorph image analysis, and Z-Y. Song for help with the graphics.

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HUMAN MUTATION 27(5), 427–435, 2006

RESEARCH ARTICLE

Intronic Alterations in *BRCA1* and *BRCA2*: Effect on mRNA Splicing Fidelity and Expression

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Communicated by Haig H. Kazazian

Germline mutations in the human breast cancer susceptibility genes *BRCA1* and *BRCA2* account for the majority of hereditary breast and ovarian cancer. In spite of the large number of sequence variants identified in *BRCA1* and *BRCA2* mutation analyses, many of these genetic alterations are still classified as variants of unknown significance (VUS). In this study, we evaluated 12 *BRCA1/2* intronic variants in order to differentiate their pathogenic or polymorphic effects on the mRNA splicing process. We detected the existence of aberrant splicing in three *BRCA1* variants (c.301-2delA/IVS6-2delA, c.441+1G>A/IVS7+1G>A, and c.4986+6T>G/IVS16+6T>G) and two *BRCA2* variants (c.8487+1G>A/IVS19+1G>A and c.8632-2A>G/IVS20-2A>G). All but one of the aberrant transcripts arise from mutations affecting the conserved splice acceptor or donor sequences and all would be predicted to result in expression of truncated BRCA1 or BRCA2 proteins. However, we demonstrated that four of these splice-site mutations (i.e., c.301-2delA, c.441+1G>A, c.4986+6T>G, and c.8632-2A>G) with premature termination codons were highly unstable and were unlikely to encode for abundant expression of a mutant protein. Three variants of *BRCA1* (c.212+3A>G/IVS5+3A>G, c.593+8A>G/IVS9+8A>G, and c.4986-20A>G/IVS16-20A>G) and four variants of *BRCA2* (c.516-19C>T/IVS6-19C>T, c.7976-4_7976_3delTT/IVS17-4delTT, c.8487+19A>G/IVS19+19A>G, and c.9256-18C>A/IVS24-18C>A) in our studies show no effects on the normal splicing process, and they are considered to be benign polymorphic alterations. Our studies help to clarify the aberrant splicing in *BRCA1* and *BRCA2* as well as provide information that can be used clinically to help counsel breast/ovarian cancer prone families. Hum Mutat 27(5), 427–435, 2006. Published 2006 Wiley-Liss, Inc.†

KEY WORDS: BRCA1; BRCA2; splicing; nonsense-mediated mRNA decay; NMD; variants of unknown significance; breast cancer; ovarian cancer

INTRODUCTION

Germline mutations in human breast cancer susceptibility genes, *BRCA1* (MIM# 113705) and *BRCA2* (MIM# 600185), are responsible for the vast majority of hereditary breast and ovarian cancer [Bove et al., 2002; Ford et al., 1998]. The lifetime risk of cancer among female *BRCA1* or *BRCA2* mutation carriers is estimated to be between 36 and 85% for breast cancer and between 16 and 60% for ovarian cancer [Breast Cancer Linkage Consortium, 1999; King et al., 2003; Malone et al., 2000]. To date, approximately 3,400 sequence variants (listed online at the Breast Cancer Information Core [BIC]; http://research.nhgri.nih.gov/bic) in each of these genes have been identified by extensive mutational analyses since their cloning and characterization in the mid-1990s [Frank et al., 2002; Miki et al., 1994; Wooster et al., 1995]. However, in spite of the large number of sequence variants in *BRCA1* and *BRCA2*, only frameshifts (insertions/deletions), splice-sites, nonsense mutations, and a few missense mutations are accepted as disease-associated genetic alterations [Breast Cancer Linkage Consortium, 1999; Frank et al., 1998; Shattuck-Eidens et al., 1997]. Little has been done with respect to other types of mutations, including intronic variants, to investigate their possible roles in the predisposition to breast and ovarian cancer [Arnold et al., 2002]. These genetic alterations, therefore, are typically classified as variants of unknown/uncertain significance (VUS). These VUSs pose a clinical challenge in the management of patients from cancer-prone families. Because of their unknown functional significance, the medical management of individuals in these families remains a dilemma.

Among these VUSs, alterations occurring in intron-exon boundaries of *BRCA1* and *BRCA2* have potential impacts on splicing fidelity. A previous study has demonstrated that variants

The Supplementary Material referred to in this article can be accessed at http://www.interscience.wiley.com/jpages/1059-7794/suppmat.

Received 2 September 2005; accepted revised manuscript 14 December 2005.

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Grant sponsor: Cheryl Herman and the Eileen Stein-Jacoby Fund, Commonwealth of Pennsylvania; Grant sponsor: National Cancer Institute; Grant number: Ovarian Cancer SPORE P50 CA83638; Grant sponsor: National Institutes of Health; Grant number: U01 CA69631; Grant sponsor: Department of Defense; Grant numbers: W81XWH-04-1-0573; DAMD17-03-1-0707; and DAMD17-03-1-0312.

DOI 10.1002/humu.20319

Published online 17 April 2006 in Wiley InterScience (www.interscience.wiley.com).

at the splicing sites affecting mRNA processing account for 15% of point mutations that associate with the genetic diseases [Krawczak et al., 1992]. Approximately 5% of all *BRCA1* and *BRCA2* alterations are splice-site mutations (http://research.nhgri.nih.gov/bic). However, only a few of these mutations have been examined for their effects on *BRCA1* and *BRCA2* mRNA splicing fidelity [Agata et al., 2003; Brose et al., 2004; Campos et al., 2003; Claes et al., 2003; Fetzer et al., 1999; Keaton et al., 2003; Laskie Ostrow et al., 2001; Pyne et al., 2000; Scholl et al., 1999] and few if any have been evaluated for their effect on the stability of the mutant transcripts.

Alterations within splicing sites of *BRCA1* and *BRCA2* also have potential impacts on mRNA transcript stability, because the mutant allele caused by aberrant splicing may contain a premature termination codon (PTC) which could activate the nonsense-mediated mRNA decay (NMD) pathway. NMD represents a phylogenetically widely conserved splicing- and translation-dependent mechanism that eliminates transcripts with PTCs and suppresses the accumulation of C-terminally truncated peptides [Frischmeyer and Dietz, 1999; Weischenfeldt et al., 2005]. Elimination of frameshift transcripts that result from aberrant splicing has been suggested as an important function of NMD [Frischmeyer and Dietz, 1999; Weischenfeldt et al., 2005]. Blocking NMD with translation inhibitor, e.g., puromycin, has been used to help detect aberrant spliced alleles which contain PTCs [Andreutti-Zaugg et al., 1997; Nomura et al., 2000].

As a result of mutation screening of *BRCA1* and *BRCA2* in hundreds of breast cancer prone kindreds, we identified six *BRCA1* and six *BRCA2* intronic alterations that are located at or near an exon-intron boundary. The purpose of this study was to characterize these 12 *BRCA1/2* variants in order to differentiate their pathogenic or polymorphic effects on mRNA splicing and expression. Our studies further defined the consequence of these sequence variants on RNA splicing and provided information regarding stability of the various mutant transcripts.

MATERIALS AND METHODS

Nomenclature and Databases

Both the Human Genetic Variation Society (HGVS) approved guidelines (www.hgvs.org/mutnomen) and the BIC (Breast Cancer Information Core; http://research.nhgri.nih.gov/bic) traditional system have been used in our studies for *BRCA1* and *BRCA2* nomenclature. In order to achieve the accuracy and also facilitate the published data comparison, an alteration was expressed as "HGVS approved/BIC Traditional" when it first appeared in the article. RefSeqs (GenBank accession no. NM_007295.2 and NM_000059.1) have been used for HGVS-approved *BRCA1* and *BRCA2* DNA and mRNA numbering, respectively. RefSeqs (GenBank accession no. NP_009226.1 and NP_000050.1) have been used for HGVS-approved BRCA1 and BRCA2 amino acid numbering, respectively. The A of the ATG translation initiation codon is +1, according to approved guidelines. RefSeqs (GenBank accession no. U14680 and U43746) have been used for BIC traditional *BRCA1* and *BRCA2* numbering, respectively.

Family Risk Assessment Program (FRAP) and *BRCA1* and *BRCA2* Mutation Analysis

Women with a family history of breast and ovarian cancer who were enrolled in the Family Risk Assessment Program (FRAP) were eligible for the present study. The FRAP was designed to provide education and cancer risk counseling for women with at least one first-degree relative (i.e., mother, sister, or daughter) with breast or ovarian cancer. Blood samples were collected following informed consent for participating in various FRAP-directed research studies including genetic susceptibility testing. Constitutional DNAs isolated from 375 participants' lymphocytes were screened for mutations in *BRCA1* and *BRCA2* as previously described [Andrulis et al., 2002; Kulinski et al., 2000; Oleykowski et al., 1998].

Lymphoblastoid Cell Lines (LCL)

Lymphocytes isolated from FRAP blood samples were infected with Epstein-Barr virus (EBV) to establish the immortal lymphoblastoid cell lines (LCLs). LCLs were maintained in RPMI media (GIBCO BRL, Gaithersburg, MD; www.gibcobrl.com) supplemented with 20% fetal calf serum (FCS) and antibiotics at 37°C, 5% CO_2 atmospheric conditions, and 95% humidity. The immortalized LCLs from cancer-free individuals who had tested negative for mutations in *BRCA1* and *BRCA2* served as wild-type controls. To prevent potential degradation of unstable transcripts by nonsense-mediated mRNA decay, a translational inhibitor, puromycin (Sigma, St. Louis, MO; www.sigmaaldrich.com), was added to the EBV cells at the concentration of 200 μg/ml for 14 hr before total RNAs were isolated.

RNA Isolation and RT-PCR

Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA; www.invitrogen.com), according to the protocols provided by the manufacturer (www.invitrogen.com/content/sfs/manuals/10296010.pdf). Purified RNA samples were further processed with DNA-free kit as suggested by the manufacturer (Ambion, Houston, TX; www.ambion.com). After quantification with a Bioanalyzer-2100 system using RNA 6000 Nano LabChip kits (Agilent Technologies, Palo Alto, CA; www.agilent.com), 2 μg of total RNA from each sample was used as a template to be reverse-transcribed (RT) in a 20-μl reaction, which also contained 5 μM random hexamers, 500 μM deoxynucleoside triphosphate mix, 1× reverse transcriptase buffer, 5 mM $MgCl_2$, 1.5 units of RNase inhibitor, and 7.5 units of MuLV RTase. All reagents were purchased from Applied Biosystems (Branchburg, NJ; www.appliedbiosystems.com). The reaction conditions were 10 min at 25°C, 1 hr at 42°C, and 5 min at 94°C. Then, 2 μl of the cDNA mixture was used in a PCR reaction, with specific primers targeting the potential splicing site. Optimal conditions were defined as: Step 1, 95°C for 10 min; Step 2, 94°C for 15 sec, 60°C for 30 sec, 72°C for 45 sec, repeated for 40 cycles; Step 3, 72°C for 7 min. The detailed information with regard to primer design and sequences are available upon request. The products were electrophoresed on 2% agarose gels. Uncloned PCR fragments were cleaned with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany; www.qiagen.com), sequenced and analyzed in both sense and antisense directions for the presence of heterozygous splicing variations. Analysis of the DNA sequences was performed using Sequencher v4.2 (Gene Codes, Ann Arbor, MI; www.genecodes.com).

Subcloning the PCR Product and Sequence Analysis

PCR products of potential mutant transcripts were purified as described above and were subcloned directly into a pCR 4-TOPO vector using the TA Cloning Kit for Sequencing (Invitrogen) following the manufacture's instruction. PCR was performed to identify bacterial colonies containing appropriate inserts. Plasmid DNA was purified using QIAfilter Plasmid Maxi Kit (Qiagen) and the insert was sequenced using either the universal M13-primers or the primers for PCR reactions.

Image Analysis and Statistical Analysis

The results of RT-PCR were quantified using the AlphaEase FC (Alpha Innotech, San Leandro, CA; www.alphainnotech.com) for the integrated density of each band. The Student's *t*-test was employed using SAS software 8.0 (SAS Institute, Cary, NC; www.sas.com). A value of $P<0.05$ was considered significant and results were presented as the mean ± standard deviation (SD).

RESULTS

BRCA1 and *BRCA2* Intronic Variants

As a result of the clinical genetic susceptibility testing of the 375 probands of breast/ovarian cancer-prone kindreds followed by FRAP, we identified several low-frequency sequence alterations in *BRCA1* and *BRCA2* that flanked the intron-exon boundaries (i.e., *BRCA1*, c.212+3A>G/IVS5+3A>G, c.301-2delA/IVS6-2delA, c.441+1G>A/IVS7+1G>A, c.593+8A>G/IVS9+8A>G, c.4986+6T>G/IVS16+6T>G, and c.4986-20A>G/IVS16-20A>G and *BRCA2*, c.516-19C>T/IVS6-19C>T, c.7976-4_7976_3delTT/IVS17-4delTT, c.8487+1G>A/IVS19+1G>A, c.8487+19A>G/IVS19+19A>G, c.8632-2A>G/IVS20-2A>G, and c.9256-18C>A/IVS24-18C>A). Supplementary Table S1A (available online at www.interscience.wiley.com/jpages/1059-7794/suppmat) lists the cancer history status of these families, and Supplementary Table S1B shows the number of times each alteration has been previously reported to the BIC database. Among these alterations, *BRCA1*-c.593+8A>G, *BRCA1*-c.441+1G>A, *BRCA2*-c.7976-4_7976_3delTT, and *BRCA2*-c.8487+19A>G are novel alterations and have not previously been reported. Five alterations, *BRCA1*-c.4986+6T>G, *BRCA1*-c.4986-20A>G, *BRCA2*-c.516-19C>T, *BRCA2*-c.9256-18C>A, and *BRCA2*-c.8632-2A>G, have been detected

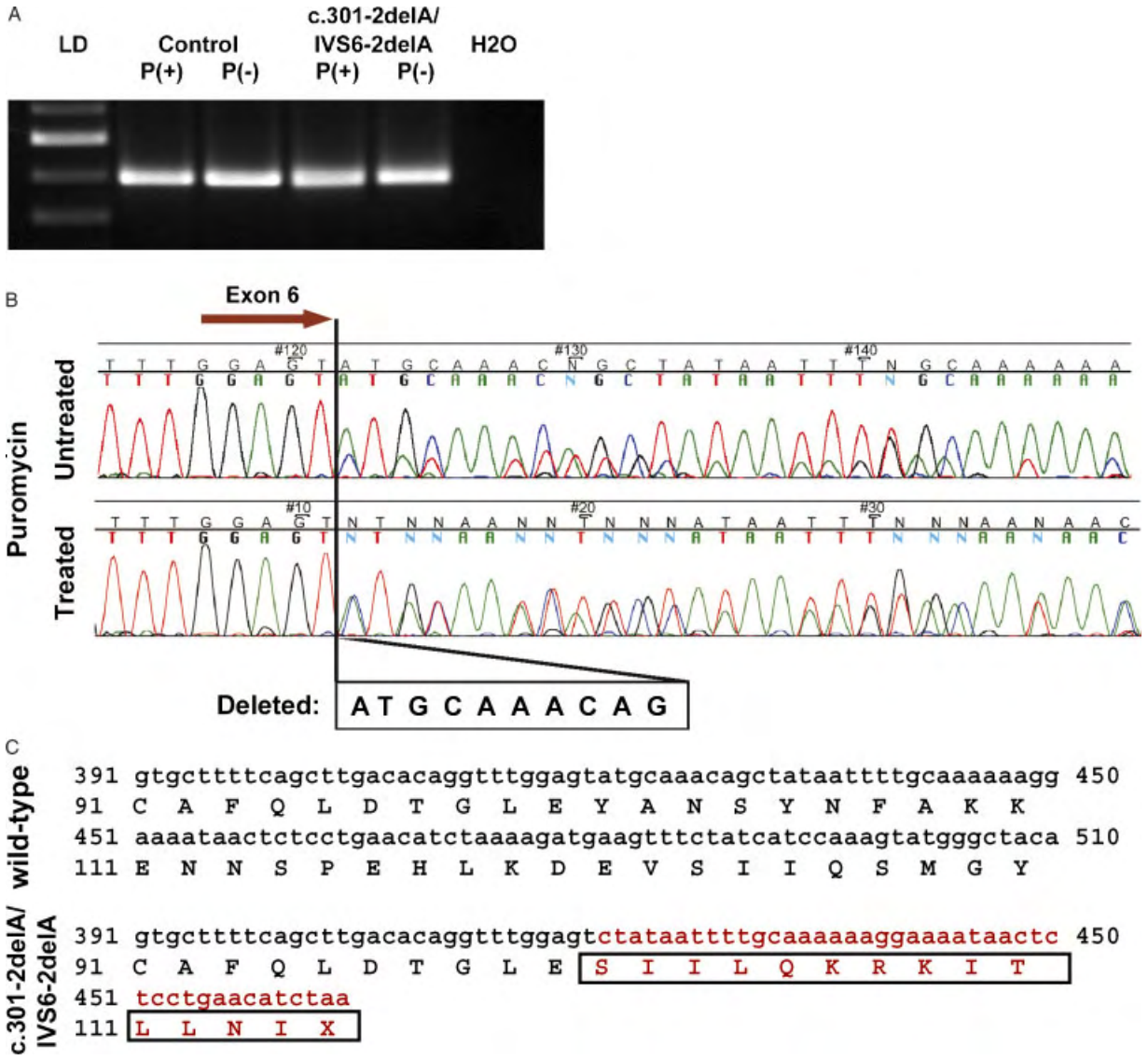

FIGURE 1. **Aberrant splicing associated with *BRCA1*-c.301-2delA/IVS6-2delA mutation. A:** RT-PCR analysis by agarose gel image. PCR samples were loaded from left to right as a DNA ladder, wild-type lymphoblastoid cells (Control) treated with puromycin (P(+)) and without puromycin (P(–)), mutation carrier lymphoblastoid cells treated with puromycin (P(+)) and without puromycin (P(–)), and negative control (H_2O). **B:** Sequencing analysis for sample from mutation carrier. *BRCA1*-c.301-2delA results in a 10-base frameshift deletion at the beginning of exon 7 (NM_007295.2:c.302_311del10). **C:** Protein sequencing analysis. The 10-bp frameshift deletion in the cDNA creates 14 new residues (101–114) and a predicted stop codon at residue 115 (NP_009226.1:p.Tyr101SerfsX15).

previously but their impact on mRNA splicing has not been investigated.

Aberrant Splicing Analysis by RT-PCR

To evaluate the consequence of each sequence variant on splicing, total RNA was isolated from cryopreserved lymphocyte pellets and/or LCLs. Primers used in the PCR reaction were designed to amplify the exon regions that are predicted to be affected by the splice-site mutations. In addition, to prevent potential degradation of unstable transcripts by nonsense-mediated mRNA decay, LCLs were treated with puromycin, a translational inhibitor, prior to RNA isolation. As shown in Supplementary Table S1B, five of the variants lead to expression of alternatively-spliced transcripts, while the other seven alterations have no apparent effects on mRNA splicing. Of these sequence variants, all but one involve the highly conserved splice donor (GT) or splice acceptor (AG) sequence flanking the intron-exon junction.

BRCA1-c.301-2delA has been reported to the BIC database, but its role in mRNA splicing has not been previously studied. In our studies, although the mutation carrier herself has no cancer, three family members have been diagnosed with breast cancer (Supplementary Fig. S1A). Analysis of cDNA products demonstrates that this intronic deletion activates another cryptic splice site, which

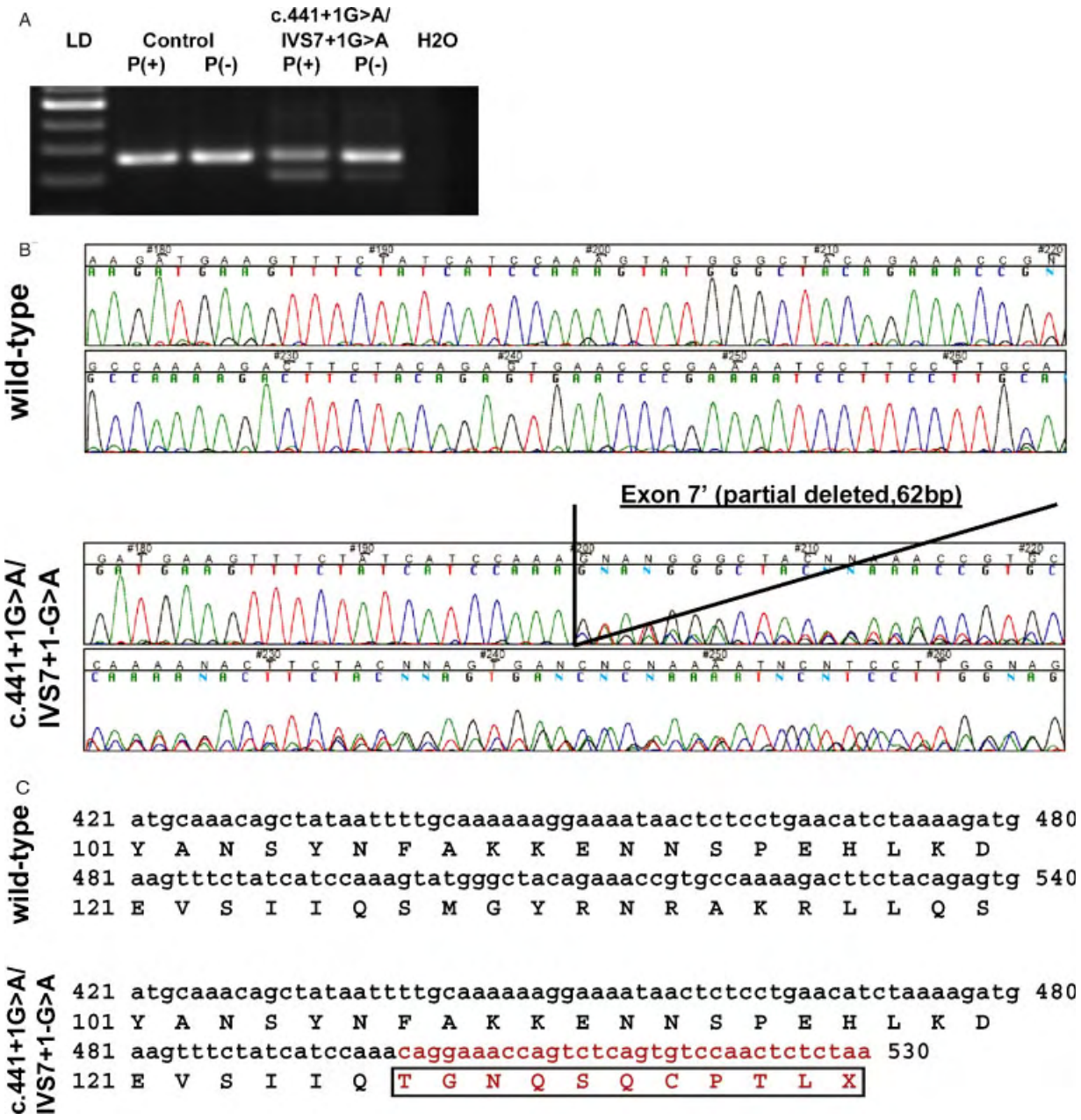

FIGURE 2. **Aberrant splicing associated with *BRCA1*-c.441+1G>A/IVS7+1G>A. A:** RT-PCR analysis by agarose gel image. PCR samples were loaded from left to right as a DNA ladder, wild-type lymphoblastoid cells (Control) treated with puromycin (P(+)) and without puromycin (P(–)), mutation carrier lymphoblastoid cells treated with puromycin (P(+)) and without puromycin (P(–)), and negative control (H_2O). **B:** Sequencing analysis for samples from wild-type and mutation carrier. The alteration *BRCA1*-c.441+1G>A. leads to a 62-bp deletion at the end of exon 7 (NM_007295.2:c.380_441del62). **C:** Protein sequencing analysis showed that this 62-bp frameshift deletion creates 10 new residues and a predicted stop codon at residue 137 (NP_009226.1:p.Ser127ThrfsX11).

results in a 10-bp frameshift deletion at the beginning of exon 7 (c.302_311del10). This frameshift deletion then results in the addition of 14 new residues (101–114) and a premature stop codon at residue 115 (p.Tyr101SerfsX15) (Fig. 1A–C). Because of only 10-bp differences between the wild-type and mutant bands, it is difficult to distinguish the wild-type and mutant bands. Therefore, the abundance of each was estimated by the peak strength in the sequence electropherograms, and the aberrant transcripts were more abundant in the puromycin treated vs. the untreated LCLs (Fig. 1B).

BRCA1-c.441+1G>A to our knowledge has not previously been reported. This alteration is detected in a proband diagnosed with breast cancer at 34 and 43 (Supplementary Fig. S1B).

TABLE 1. **Ratio of mRNA Expression of the Aberrant Spliced Alleles to the Wild-Type Alleles in LCLs Treated With or Without Puromycin**

	BRCA1			BRCA2	
	C.301-2delA[a]/ IVS6-2delA[b]	c.441+1G>A/ IVS7+1G>A	c.4986T>G/ IVS16+6T>G	c.8487+1G>A/ IVS19+1G>A	c.8632-2A>G/ IVS20-2A>G
Nontreated ±0.05[d]	–[c]	0.35±0.08	0.29±0.08	1.96±0.56	0.72±0.02/0.54
Treated ±0.15	–	0.69±0.06	0.59±0.10	1.97±0.56	0.96±0.13/1.00
P-value	–	0.03	0.03	NS	0.05/0.005

[a]RefSeqs (GenBank accession no. NM_007295.2 and NM_000059.1) are used for *BRCA1* and *BRCA2* numbering, respectively, and the A of the ATG translation initiation codon is +1, according to approved guidelines (www.hgvs.org/mutnomen).
[b]Traditional nomenclature used in BIC database, based on RefSeqs (GenBank accession no. U14680 and U43746) for *BRCA1* and *BRCA2* numbering, respectively.
[c]Because of 10-bp differences between the wild-type and mutant bands, the density was not able to be quantified.
[d]It represents the ratios of two aberrant spliced alleles to the wild-type alleles, NM_000059.1:c.[8632-1356_8632-1264ins; 8633_8675del] (upper band in Fig. 5A) and NM_000059.1:c.8633_8675del (lower band in Fig. 5A), respectively.

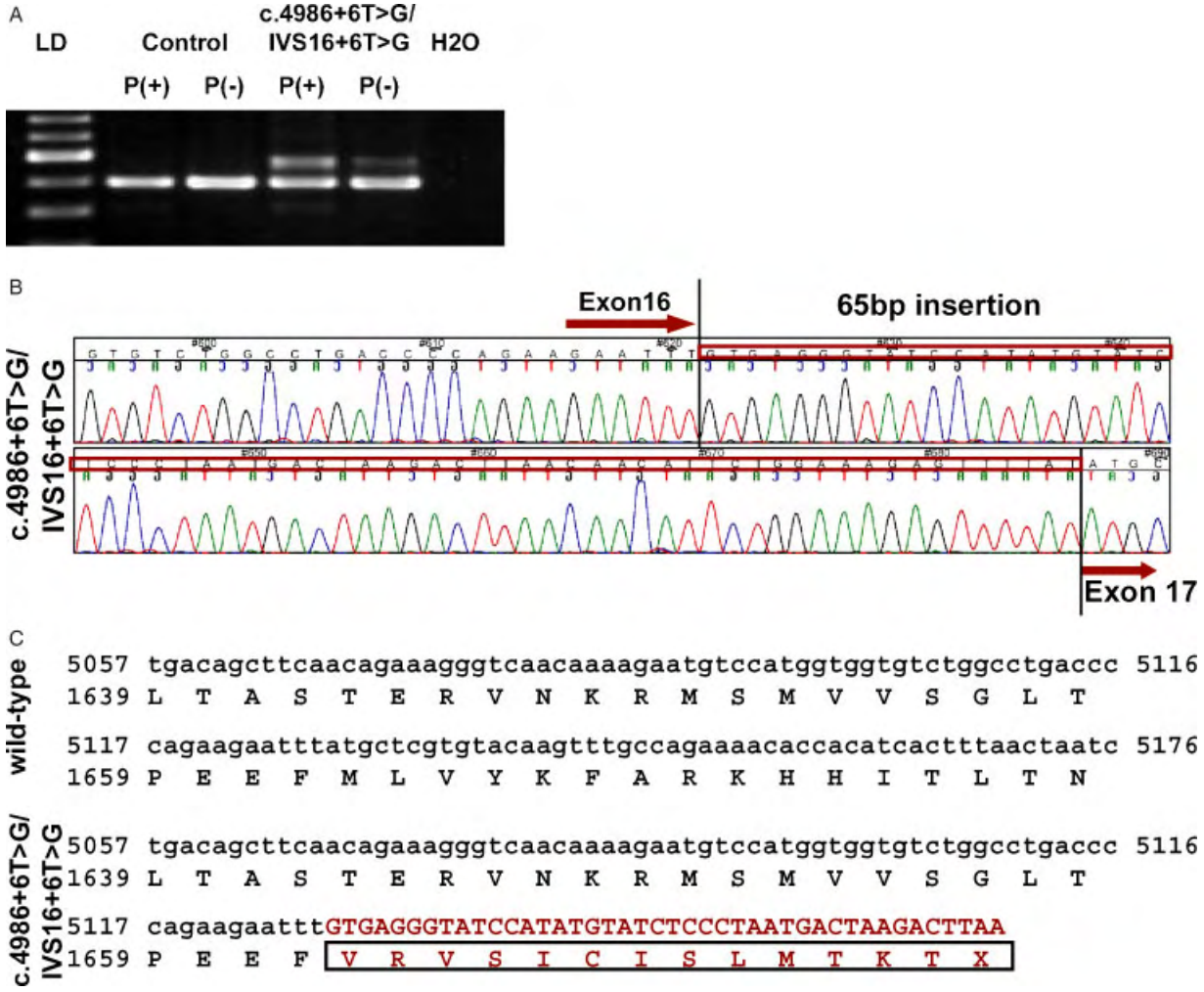

FIGURE 3. **Aberrant splicing associated with *BRCA1*-c.4986+6T>G/IVS16+6T>G. A:** RT-PCR analysis by agarose gel image. PCR samples were loaded from left to right as a DNA ladder, wild-type lymphoblastoid cells (Control) treated with puromycin (P(+)) and without puromycin (P(–)), mutation carrier lymphoblastoid cells treated with puromycin (P(+)) and without puromycin (P(–)), and negative control (H_2O). **B:** Sequencing analysis for samples from mutation carrier following subcloning into pCR4-TOPO. This alteration leads a 65-base insertion from intron 16 (NM_007295.2:c.4916+1_4916+65ins65). **C:** Protein sequencing analysis indicate this 65-bp insertion creates a predicted stop codon at residue 1676 (NP_009226.1:p.Met1663ValfsX14).

The inheritance of this mutation is unclear, since both the maternal and paternal side of the family report breast or ovarian cancer and no other samples were available from this kindred. Analysis of mRNA from the lymphocytes of this individual by RT-PCR showed that this alteration results in a 62-base deletion within exon 7 (c.380_441del62) (Fig. 2A–C). This frameshift is predicted to result in the addition of 10 amino acid residues and premature termination of translation at residue 137 (p.Ser127ThrfsX11) (Fig. 2C). The aberrant transcript is presented in both puromycin treated and untreated LCLs. However, the mRNA expression ratios of mutant alleles to wild-type alleles are approximately 0.4:1 and 0.7:1 in puromycin untreated and treated LCLs, respectively ($P < 0.03$) (Fig. 2A; Table 1).

BRCA1-c.4986+6T>G has been reported previously, but has not been functionally characterized. The proband was diagnosed with ovarian cancer at age of 62, and reported a strong family history of breast and ovarian cancer (Supplementary Fig. S1C). We performed RT-PCR to amplify the region from exon 17 to 22 using RNA samples isolated from peripheral lymphocytes of the alteration carrier and cancer-free controls and demonstrated that this alteration leads to an aberrant *BRCA1* transcript, which incorporates 65 bases of intron 16 sequence, immediately following exon 16 (c.4916+1_4916+65ins65) (Fig. 3A–C). Sequence analysis indicates the mutation results in the activation of a cryptic splice site at g.38,476,405 (GenBank accession no. NC_000017.9). This 65-base insertion is predicted to create a stop codon at residue 1676, plus 13 additional residues encoded by the inserted sequence (p.Met1663ValfsX14). The mRNA expression ratios of mutant alleles to wild-type alleles are approximately 0.3:1 and 0.6:1 in puromycin untreated and treated LCLs, respectively ($P < 0.03$) (Fig. 3A; Table 1).

BRCA2-c.8487+1G>A has been previously reported to the BIC database. For this family, the proband was diagnosed with invasive breast cancer at age 41, and she reported two paternal aunts and a paternal grandmother with breast cancer (Supplementary Fig. S1D). RT-PCR analysis of RNA from the proband's peripheral lymphocytes found that exon 19 (156 bp) was deleted (c.8332_8487del156), resulting in an in-frame deletion of 52 amino acids (p.Ile2778_Gln2829del52) (Fig. 4A and B). Interestingly, the level of the mutant *BRCA2* transcript appears to be much more abundant than the wild-type transcript. The mRNA expression ratios of mutant alleles to wild-type alleles are almost identical (1.96:1 vs. 1.97:1) in puromycin untreated and treated LCLs, respectively (Fig. 4A; Table 1).

BRCA2-c.8632-2A>G has been reported one time previously to BIC. The proband in this family was diagnosed with ovarian cancer at age 39 and reported two sisters with breast cancer at 34 and 35 years of age (Supplementary Fig. S1E). As before, we used RT-PCR to amplify the region from exon 19 to 22. We found that the *BRCA2*-c.8632-2A>G mutation leads to two splicing variants, a 43-bp deletion at the beginning of exon 21 (c.8633_8675del43) or a 93-bp insertion from intron 20 in

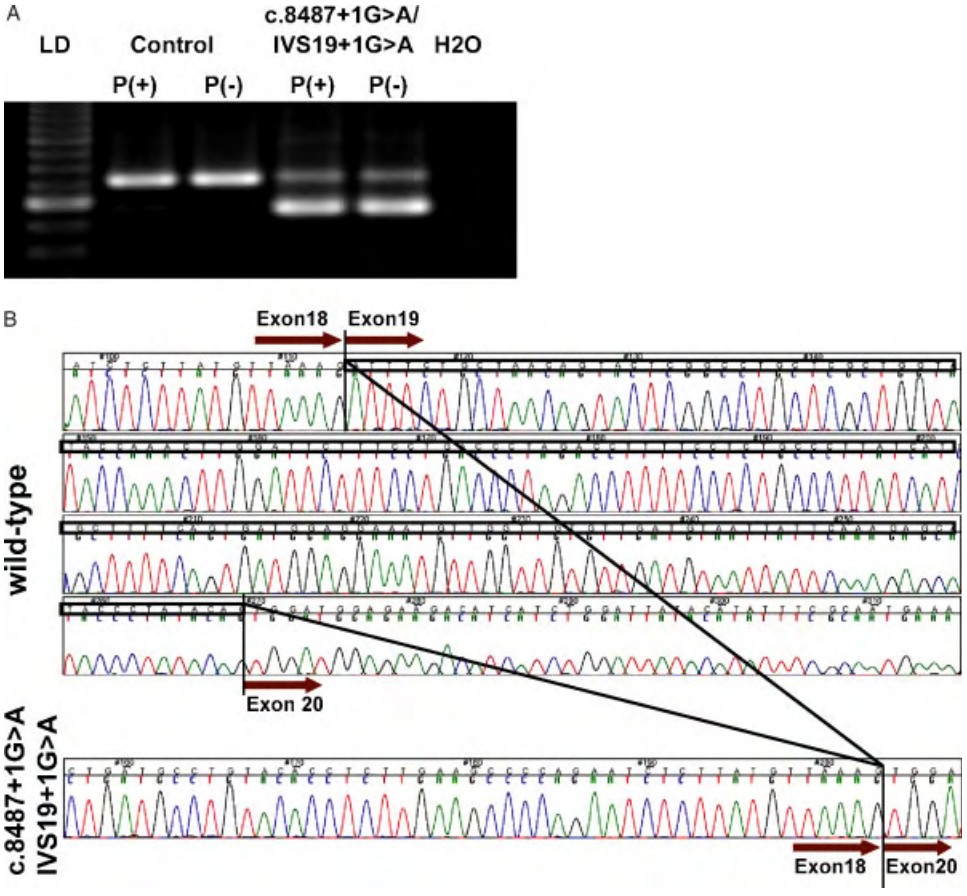

FIGURE 4. **Aberrant splicing associated with *BRCA2*-c.8487+1G>A/IVS19+1G>A. A: RT-PCR analysis by agarose gel image. PCR samples were loaded from left to right as a DNA ladder, wild-type lymphoblastoid cells (Control) treated with puromycin (P(+)) and without puromycin (P(–)), mutation carrier lymphoblastoid cells treated with puromycin (P(+)) and without puromycin (P(–)), and negative control (H_2O). B: Sequencing analysis for samples from wild-type and mutation carrier by subcloning. *BRCA2*-c.8487+1G>A allele skips exon 19 (NM_ 000059.1:c.8332_8487del156).**

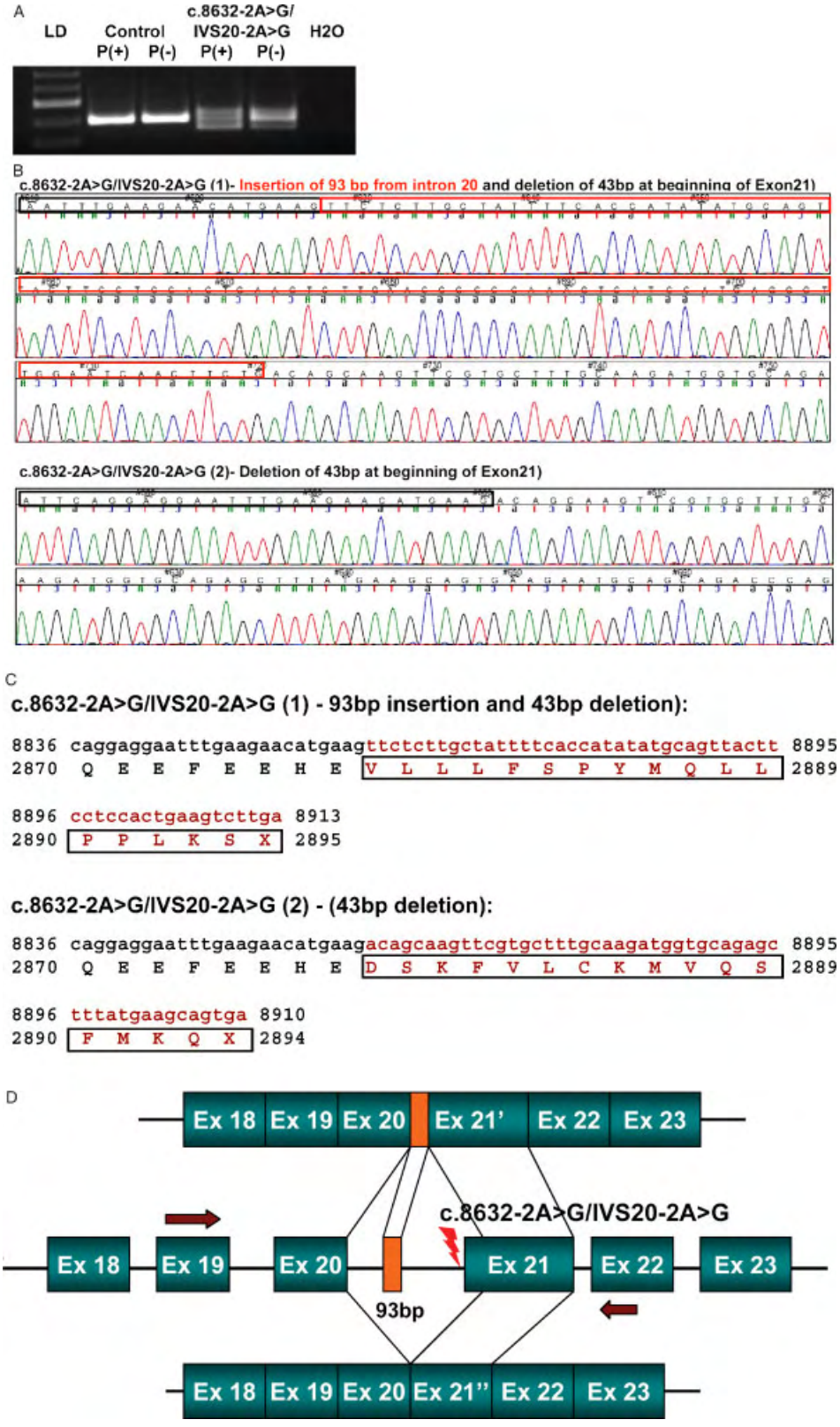

FIGURE 5. **Aberrant splicing associated with *BRCA2*-c.8632–2A>G/IVS20–2A>G. A:** RT-PCR analysis by agarose gel image. PCR samples were loaded from left to right as a DNA ladder, wild-type lymphoblastoid cells (Control) treated with puromycin (P(+)) and without puromycin (P(–)), mutation carrier lymphoblastoid cells treated with puromycin (P(+)) and without puromycin (P(–)), and negative control (H_2O). **B:** Sequencing analysis for samples from mutation carrier by subcloning. The alteration of *BRCA2*-c.8632–2A>G leads to either the 93-bp insertion from intron 20 and the 43-bp deletion of exon 21 (NM_ 000059.1:c.[8632-1356_8632-1264ins93; 8633_8675del43]), or the 43-bp deletion at the beginning of exon 21 (NM_ 000059.1:c.8633_8675del43). **C:** Protein sequencing analysis indicates that *BRCA2*-c.8632-2A>G causes two distinct BRCA2 truncated proteins, NP_000050.1:p.Glu2878ValfsX18 and p.Glu2878AspfsX17. **D:** Schematics for aberrant splicing caused by mutation *BRCA2*-c.8632–2A>G.

combination with the 43-bp deletion of exon 21 (c.[8632-1356_8632-1264ins93; 8633_8675del43], GenBank Accession No: NC_000013.9 and NM_000059.1), which are both predicted to lead to truncated proteins, p.Glu2878ValfsX18 and p.Glu2878AspfsX17, respectively (Fig. 5A–D). The mRNA expression ratios of mutant alleles to wild-type alleles (c.[8632-1356_8632-1264ins93; 8633_8675del43], i.e., upper band) is approximately 0.7:1 and 1:1 in puromycin untreated and treated LCLs, respectively ($P < 0.05$). The mRNA expression ratio of mutant alleles to wild-type alleles (c.8633_8675del43, i.e., lower band) is 0.5:1 and 1:1 in puromycin untreated and treated LCLs, respectively ($P < 0.005$) (Fig. 5A; Table 1).

DISCUSSION

Since *BRCA1* and *BRCA2* were cloned and characterized in the mid-1990s [Frank et al., 2002; Miki et al., 1994; Wooster et al., 1995], hundreds of different sequence variants in each of these genes have been detected, including a large number of the intronic variants. We characterized the potential pathological significance of 6 *BRCA1* and 6 *BRCA2* intronic alterations at the mRNA level. All of the sequence alterations are located within or near consensus splicing sites. We detected aberrantly spliced transcripts for three of the *BRCA1* variants (c.301-2delA, c.441+1G>A/IVS7+1G>A, and c.4986+6T>G) and two of the *BRCA2* variants (c.8487+1G>A and c.8632-2A>G). The other *BRCA1* and *BRCA2* variants show no effects on RNA splicing.

In our efforts to classify *BRCA1* and *BRCA2* variant transcripts, we found that treatment of LCLs with puromycin helped facilitate the analysis of the mutant transcripts. Because of potential degradation of unstable transcripts by mRNA NMD [Losson and Lacroute, 1979; Perrin-Vidoz et al., 2002], the aberrant transcripts identified in the LCLs treated with puromycin may be considerably less abundant or even absent in the nontreated LCLs and peripheral lymphocytes. Except for the aberrant transcripts associated with the *BRCA2*-c.8487+1G>A, the other aberrant transcripts identified in our study are weakly expressed (*BRCA1*-c.301-2delA, *BRCA1*-c.441+1G>A, *BRCA1*-c.4986+6T>G, and *BRCA2*-c.8632-2A>G) in comparison to the wild-type transcripts (Table 1). The common characteristics shared by these four alterations are that they all result in a frameshift in the translational reading frame and a PTC. In comparison, *BRCA2*-c.8487+1G>A is predicted to result in a 52-amino acid in-frame deletion that does not cause a PTC (Fig. 4A and B). Furthermore, we examine the NMD rule—"PTCs before ~50–55-bp of the end of the penultimate exon initiate NMD"—and all the PTCs containing alleles in our studies comply with this 50–55 nucleotide rule (data not shown). These results suggest that the mutated *BRCA1* and *BRCA2* transcripts that create premature stop codons are less stable. Therefore, *BRCA1* and *BRCA2* aberrant transcripts eliminated by NMD could result in downregulating the overall level of both mRNA and protein of BRCA1 and BRCA2. Loss of BRCA1 expression has been reported to be related to the pathogenesis of breast cancer [Thompson et al., 1995; Wilson et al., 1999; Zheng et al., 2000; Wei et al., 2005].

In conclusion, our study has evaluated several *BRCA1* and *BRCA2* variants located within or near intron-exon boundaries. Seven of them have no apparent impact on the mRNA splicing process, four cause frameshift mutation, and one resulted in an in-frame deletion. These studies help to confirm whether alterations in *BRCA1* and *BRCA2* are likely to be benign polymorphisms or pathogenic mutations. As our study is fundamentally based on the analysis of mRNA transcripts, it will be helpful to carry out further research at the protein level since many of the mutant alleles could be reduced in expression relative to wild-type indicating that loss of BRCA1 or BRCA2 protein may contribute to the pathogenesis of breast and/or ovarian cancer rather than expression of a defective protein.

ACKNOWLEDGMENTS

This work was supported in part by Breast Cooperative Family Registry grant U01 CA69631 (to M.B.D.), a fellowship from the Department of Defense (W81XWH-04-1-0573 to X.C.), and Department of Defense grants (DAMD17-03-1-0707 and DAMD17-03-1-0312 to A.K.G.).

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Review

BRCA1-associated complexes: new targets to overcome breast cancer radiation resistance

Xiaowei Chen†, Cletus A Arciero and Andrew K Godwin

Since BRCA1 was cloned a decade ago, significant progress has been made in defining its biochemical and biological functions, as well as its role in breast and ovarian cancers. BRCA1 has been implicated in many cellular processes, including DNA repair, cell cycle checkpoint control, protein ubiquitination and chromatin remodeling. This review examines the role(s) of BRCA1 in mediating these cellular processes, and discusses its potential involvement in the resistance of breast cancer to radiation-based therapies. Finally, the possibility that BRCA1-associated proteins may serve as new targets for breast cancer radiation therapy is explored. The activation or inactivation of these BRCA1-associated proteins may modify both the risk of developing cancers in *BRCA1* mutation carriers and the efficacy of breast cancer therapy, including radiation.

Expert Rev. Anticancer Ther. 6(2), 187–196 (2006)

CONTENTS

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KEYWORDS:
BRCA1, breast cancer, cell cycle, chromatin remodeling, DNA repair, protein interaction, radiation resistance, ubiquitination

Breast cancer is the most common cancer affecting women, with a lifetime risk of approximately 10% by the age of 80 years. In the USA, it is estimated that in 2005 there was approximately 211,000 new cases of breast cancer, and more than 40,000 breast cancer-related deaths [1]. Inherited mutations in the breast cancer susceptibility gene (*BRCA1*) and *BRCA2* predispose women to high risks of breast and ovarian cancer [2]. Lifetime risks of breast cancer are as high as 80% among American women with mutations in these genes; lifetime risks of ovarian cancer are more than 40% for *BRCA1* and 20% for *BRCA2* mutation carriers [2]. Risks for young women with inherited *BRCA1* or *BRCA2* mutations are particularly increasing. Among Caucasian women in the USA, between 5 and 10% of breast and ovarian cancer cases are due to inherited mutations in *BRCA1* and *BRCA2* [3,4]. Mutations in other genes also influence breast cancer risk, including *CHEK2*, *TP53* (associated with Li–Fraumeni syndrome) and *PTEN* (associated with Cowden syndrome) [5,6].

Since its cloning and characterization in the mid-1990s [7], BRCA1 has been implicated in many cellular processes including DNA repair, cell cycle checkpoint control, protein ubiquitination and chromatin remodeling. Despite the fact that *BRCA1* mutations contribute to hereditary breast and ovarian cancer, it is surprising that *BRCA1* is rarely found to be mutated in sporadic breast cancers, which account for approximately 90% of all breast cancers [8]. Nevertheless, studies have indicated that loss of *BRCA1* expression through epigenetic mechanisms may contribute significantly to sporadic breast cancer [9,10]. In addition, evidence is accumulating that dysfunction of other genes coding for proteins in complementary pathways to BRCA1, might be important in the pathogenesis of a significant proportion of sporadic, nonfamilial cancers. This speculation comes from several lines of evidence, including both phenotypic analyses of breast and ovarian tumors as well as mechanistic studies of BRCA1-associated pathways [11,12].

BRCA1 has been implicated in normal cellular processes, including DNA fidelity and damage repair, and has therefore been examined as having a possible role in the radioresistance of breast tumors. Breast cancer cell line and animal model studies have shown an increased sensitivity to ionizing radiation when BRCA1 is defective [13–17]. This review

10.1586/14737140.6.2.187

ISSN 1473-7140

will provide an update on what is currently known about the molecular and cellular functions of BRCA1, and speculate on how these functions contribute to the pathogenesis of breast cancer.

BRCA1: a breast cancer susceptibility gene

Genetic transmission of an autosomal dominant factor responsible for familial breast carcinoma was first reported in the early 1970s [18]. Hall and colleagues made significant progress in breast cancer molecular genetics during the 1990s by utilizing gene linkage studies, which identified an association between a locus on chromosome 17q21 and early onset familial breast cancer [19]. *BRCA1* was isolated in 1994 and is composed of 23 exons (22 of which are coding) that are distributed over approximately 80 kbp of genomic DNA (GenBank reference: NM_007295.2 and NC_000017.9). The 7.4-kb transcript is detected in numerous tissues, including breast and ovary, and encodes a predicted protein of 1863 amino acids. Many alternatively spliced transcript variants have been reported for *BRCA1*, but the significance of these in regards to function is not well known as only a few code for a protein that has been detected and studied functionally. Nevertheless, several groups have reported that some of the alternative spliced forms of *BRCA1* may influence its subcellular localization, as well as its ability to interact with other proteins, and thus, its physiological functions [20,21]. Interestingly, BRCA1 shows little homology to other known proteins except for 42 amino acids at the amino terminus that encode a really interesting new gene (RING)-finger, a motif found in a subset of proteins that interact with nucleic acids and/or form protein–protein complexes [22]. In addition, a region of BRCA1 encoded by exon 11 (amino acids 200–300) possesses a nuclear localization signal and sequences that serve as docking sites for many critical cellular proteins [23]. Furthermore, BRCA1 contains two BRCA1 C-terminal (BRCT) domains in its C-terminal region (amino acids 1650–1860) (FIGURE 1) [24]. The BRCT motif of BRCA1 binds to many proteins, including RNA polymerase (RNAP) II, p300, BACH1, p53 and retinoblastoma (RB), and plays an important role in DNA repair and/or cell cycle checkpoints [24].

Since the identification of *BRCA1*, there has been a tremendous amount of effort focusing on the genetic characterization of this gene and the related culprit breast susceptibility gene, *BRCA2*. As a result of mutation screening that has spanned the globe and all nationalities afflicted with hereditary breast and ovarian cancer, more than 1500 sequence variants [101] have been detected throughout *BRCA1* (FIGURE 1). In spite of the large number of sequence variants in *BRCA1*, only frameshift mutations (insertions/deletions, some of which can be several kbp in length), splice site mutations (that lead to aberrant mRNA splicing and disrupt the generation of the normal protein product), nonsense mutations (single bp substitutions that result in premature termination of protein translation and, therefore, expression of a truncated protein) and a few missense changes (a single bp exchange that results in an amino acid substitution) are accepted as disease-associated genetic alterations [25,26]. Little research has been performed regarding other types of mutations, primarily intron variants and missense changes in coding sequence, in order to investigate their potential roles in the predispositions to breast and ovarian cancers [27,28]. These genetic alterations are therefore typically classified as variants of unknown/uncertain significance (VUS). These VUSs pose a major clinical challenge in the management of patients from cancer-prone families, since an 'indeterminate' result frequently leaves both the genetic counselors and patients searching for more answers. Currently, few of these VUSs have been proven experimentally to be deleterious and disease causing. It is likely that the vast majority of VUSs will ultimately be proven to be benign polymorphisms or variants associated with a modest increase in cancer risk.

Function of *BRCA1*: a puzzle not fully solved

BRCA1 is one of the most intensively studied genes in the breast cancer research field due to its clinical importance. The *BRCA1* gene encodes for a 220-kDa nuclear phosphoprotein that has been suggested to play a role in maintaining genomic stability and to act as a tumor suppressor. BRCA1 interacts directly or indirectly with other tumor suppressors (such as p53 and BRCA2), DNA damage sensors (such as RAD51, RAD50, MRE11 and NBS1) and signal transducers (such as p21 and cyclin B) to form multisubunit protein complexes, such as

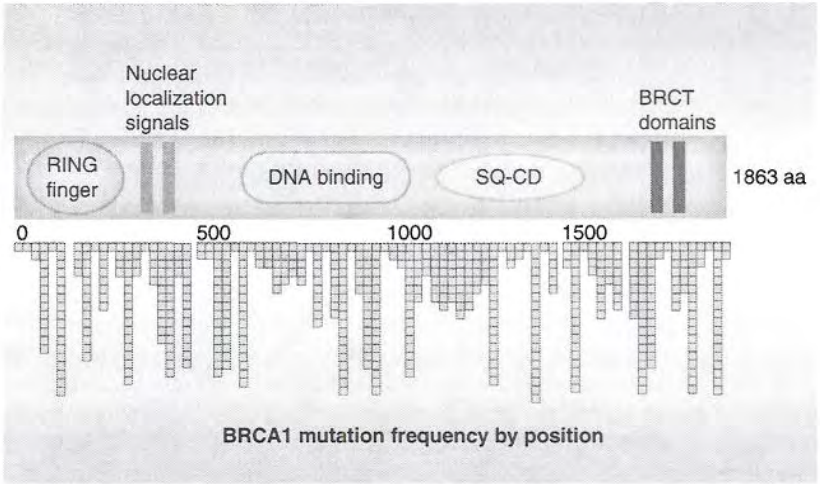

Figure 1. Schematic of the functional domains of BRCA1 and mutation frequency graph. The *BRCA1* gene codes a protein with 1863 amino acids and five protein functional domains. The five domains are the RING finger, nuclear localization signal, DNA binding domain, SQ (serine and threonine)-cluster domain (CD) and BRCA1 carboxyl terminus (BRCT) domain. The height of the vertical bar presents the relative mutation frequency across the BRCA1 protein. [Modified from Breast Cancer Information Core (BIC) database].

BRCA1-associated genome surveillance complex (BASC) and BRCA1 and BRCA2 containing complex (BRCC). These multisubunit protein complexes are involved in a broad range of biological processes including DNA repair, cell cycle control, ubiquitination and chromatin remodeling. However, the number of these BRCA1 protein-associated complexes and their complexity has yet to be fully elucidated. Thus, much of the current scientific effort involving BRCA1 is being directed at defining the biochemical functions of BRCA1 and its protein interactions.

BRCA1 cellular localization

The BRCA1 protein undergoes hyperphosphorylation during late G_1 and S phases of the cell cycle [29]. The nuclear localization signal motif (amino acids 200–300) appears to serve as a docking site for importin-α, a subunit of the nuclear transport signal receptor [23]. This physical interaction has led to the speculation that BRCA1 may be involved in the nuclear localization sequence (NLS) receptor-mediated pathway of nuclear import and play a role in recruiting other proteins to the DNA double-strand break (DSB) site following DNA damage [30]. In proliferating cells, BRCA1 forms discrete nuclear foci (dots) during the S phase as well as the G_2 phase of the cell cycle. Loss of the BRCA1 foci in subsequent phases of the cell cycle is accompanied by a specific, dose-dependent change in the state of BRCA1 phosphorylation [31]. Following cellular exposure to ionizing radiation, UV or various chemotherapeutic agents, such as cisplatin, BRCA1 becomes phosphorylated and forms discrete nuclear foci in response to DNA damage [31]. These observations continue to indicate that phosphorylation of BRCA1 is a critical step for dynamic BRCA1 nuclear localization during cell proliferation and the cellular response to DNA damage.

Role of BRCA1 in DNA repair

The majority of BRCA1 functional studies have focused on its potential role in response to DNA damage. The direct implication of BRCA1 as a component of DNA damage response pathways comes from its interactions with BRCA2 and RAD51. BRCA1, BRCA2 and RAD51 form a protein complex that activates DSB repair and initiates homologous recombination, which links the maintenance of genomic integrity to tumor suppression [32]. In addition, Scully and colleagues found that cells expressing exogenous wild type BRCA1 were less sensitive to γ-irradiation and were more efficient in repairing DSBs compared with a BRCA1-deficient breast cancer line, HCC1937 [33]. In animal models, impaired repair of chromosomal DSBs by homologous recombination have been noted in BRCA1-deficient mouse embryonic stem cells [34]. Furthermore, increased levels of chromosomal damage have been reported in lymphocytes following irradiation exposure in *BRCA1* mutation carriers [35]. Recent studies indicate that BRCA1 is also involved in nonhomologous end joining. *BRCA1* mutants make this error-prone repair mechanism even less accurate, which could lead to chromosomal rearrangement and instability [36,37]. In addition, BRCA1 has been shown to have a role in nucleotide-excision repair. BRCA1 specifically enhances the global genomic repair (GGR) pathway by inducing the expression of nucleotide excision repair (NER) genes, including XPC, DDB2 and GADD45, independent of p53 [38].

Role of BRCA1 in cell cycle control

BRCA1 has been shown to stimulate expression of the cyclin-dependent kinase (CDK) inhibitor, p21, and to inhibit cell cycle progression into S phase [39]. In addition, researchers have shown that BRCA1 is not only essential for activating the CHEK1 kinase that regulates G_2/M arrest induced by DNA damage, but also controls the expression, phosphorylation and cellular localization of Cdc25C and Cdc2/cyclin B kinases [40]. Therefore, BRCA1 appears to be involved in regulating the onset of mitosis. In mouse knockout models where the *BRCA1* gene is mutated in such a way that BRCA1 protein is not functional, $BRCA1^{-/-}$ (null) embryos fail to fully develop. However, if either *Tp53* or *p21* are also eliminated by genetic approaches, *BRCA1* mutant embryos ($BRCA1^{-/-}$; $Tp53^{-/-}$ or $BRCA1^{-/-}$; $p21^{-/-}$) show prolonged survival from embryonic days 7.5 to 9.5 [41]. In addition, a defective G_2/M checkpoint and extensive chromosomal abnormalities have been found in cells from other *BRCA1* knockout mice in which exon 11 was removed [42]. It is also reported that elimination of one *Tp53* allele ($Tp53^{+/-}$; $BRCA1^{exon11-/-}$) rescued this embryonic lethality caused by the deletion of *BRCA1* exon 11 and restored normal mammary gland development [43]. However, most female mice homozygous for the *BRCA1* exon 11 deletion and heterozygous for loss of the *Tp53* gene developed mammary tumors within 6–12 months. Importantly, the resulting tumors lost the remaining *Tp53* allele [43]. These findings indicate that the genetic interactions between *BRCA1* and *p53* are associated with breast carcinogenesis. Moreover, primary fibroblasts, which are heterozygous for a *BRCA1* mutation, display an abnormal G_1/S cell cycle checkpoint following UVA irradiation [44].

Role of BRCA1 in ubiquitination

The RING-finger motif in the N-terminus of BRCA1 has been found to be the site of heterodimerization of BRCA1 and BRCA1-associated RING domain 1 (BARD1). BARD1 is another RING-finger-containing protein that was identified using the BRCA1 RING-finger domain as the 'bait' in a protein interaction assay (yeast two-hybrid screen) [45]. When bound to BARD1, BRCA1 shows significant ubiquitin ligase activity and is capable of polymerizing ubiquitin [46]. Importantly, deleterious mutations affecting the BRCA1 RING-finger domain, found in clinical specimens, abolish the ubiquitin ligase activity of BRCA1 [16,45]. These findings support a relationship between BRCA1's ligase activity and a predisposition to breast cancer.

BRCA1 interacts with a number of proteins and functions in diverse cellular processes. Therefore, it is not surprising that BRCA1's ubiquitin ligase activity may contribute to more than one of the biological roles of BRCA1. BRCA1 has also been reported to interact with the RNAP II holoenzyme [47]. Two recent reports have suggested that the BRCA1/BARD1 complex may be involved in the degradation of RNAP complex,

and siRNA-mediated knockdown of BRCA1 and BARD1 results in stabilization of RNAP II in the cells following UV exposure [48,49]. These studies reported that BRCA1/BARD1 appears to initiate the degradation of stalled RNAP II and, thus, disrupt the coupled transcription by inhibiting RNA processing machinery in cells exposed to DNA damage. At present, the known substrates that are polyubiquitinated by the BRCA1–BARD1 ubiquitin ligase are very limited and include RNAP II, nucleophosmin/B23 and p53 [48–51].

Role of BRCA1 in chromatin remodeling

Wang and colleagues used immunoprecipitation and mass spectrometry to identify a large multisubunit protein complex, BASC, in which the DNA repair proteins identified include ATM, BLM, MSH2, MSH6, MLH1, the RAD50–MRE11–NBS1 complex and the RFC1–RFC2–RFC4 complex [52]. Confocal microscopy demonstrated that BRCA1, BLM and the RAD50–MRE11–NBS1 complex colocalized to large nuclear foci, and BASC has subsequently been shown to be involved in chromatin remodeling at sites of DSBs [52]. In addition, BRCA1 directly interacts with the brahma-related gene 1 (BRG1) subunit of SW1/SNF-associated complex, which is involved in chromatin remodeling [53]. This finding links chromatin remodeling processes to breast cancer. Furthermore, the BRCT domain of BRCA1 has been reported to be associated with the histone deacetylases, HDAC1 and HDAC2 [54]. All of these findings help explain the involvement of BRCA1 in multiple, seemingly unrelated processes such as transcription and DNA repair.

BRCA1-associated proteins: targets for breast cancer radiation therapy

In the past several decades, efforts have been made toward understanding the mechanisms of the response to both cytotoxic chemotherapy and ionizing radiation breast cancer treatment. Owing to the important role of BRCA1 in DNA repair, cell cycle control and other pathways, breast tumors with defective BRCA1 are likely to be more sensitive to chemotherapy and ionizing radiation. Although both chemotherapy and radiation have been extensively studied in the clinical treatment of breast cancer, the following section will explore the possibility that BRCA1 and its interacting proteins could become novel targets in enhancing breast cancer radiation therapy. The role of BRCA1 in chemotherapy has been reviewed comprehensively by other groups [55,56].

Radiation therapy for breast cancer

Radiation therapy has been a treatment modality for breast cancer patients for more than 100 years. Over the past 30 years, radiation therapy has become a critical step in the successful treatment of breast cancer. The role of radiation therapy grew in the early 1970s, when Fletcher documented its instrumental role in decreasing local recurrences [57]. In particular, supraclavicular metastases were reduced from 20–25% to 1.3–3% with the addition of ionizing radiation. Radiation therapy was also used to treat patients with tumors that had undergone total mastectomy, producing a decrease in local recurrences of over two-thirds [57]. This early work led to an expanded role for radiation therapy in breast cancer.

The emergence of radiation therapy to the forefront of modern breast cancer treatment lies in its application in breast-conservation therapy. Landmark studies on the necessity of radiation therapy in breast-conservation therapy were performed by Fisher and colleagues, as a part of the National Surgical Adjuvant Breast and Bowel Project (NASBP) B-06 trial, which demonstrated that lumpectomy with radiation therapy had much lower recurrence rates than lumpectomy alone (14 vs 39%) [58]. This observation has been further validated by an extensive meta-analysis that supported improved local control with the addition of radiation therapy [59,60].

Recently, clinical research has examined the possible survival benefits of radiation therapy in breast cancer. The Early Breast Cancer Trialists' Collaborative Group (EBCTG) examined 78 trials involving more than 42,000 patients with breast cancer [61]. In the analyses of trials directly comparing patients receiving radiation therapy with those not receiving radiation therapy, a clear reduction in local recurrences was again seen in the radiotherapy group, including patients undergoing mastectomy or breast-conservation therapy [61]. Interestingly, there was also a noted improvement in survival in patients treated with radiotherapy. In fact, patients receiving radiotherapy for breast cancer had a 5.4% reduction in their 15-year breast cancer mortality risk and a 4.4–5.3% reduction in overall mortality [61]. These findings support the observation that radiotherapy contributes to not only the reduction of local recurrences, but also the reduction in 15-year overall mortality.

Despite the benefits of radiation therapy in the treatment of breast cancer, patients still develop local recurrences in the targeted breast. Researchers have noted that breast cancer recurrences in the nonirradiated breast often occur within 3 years of initial diagnosis [62]. In comparison, local recurrences in irradiated breast tissue occur much later, and the risk increases with time (7, 14 and 20% risk at 5, 10 and 20 years, respectively) [63]. It is these recurrences that have spurred research in both breast cancer recurrences and the possibility of radioresistant breast tumors.

BRCA1 in resistance to breast cancer resistance therapy

The damage caused by ionizing radiation activates DNA damage cell cycle checkpoints leading to the various DNA repair pathways. The central component of these pathways is the ATM/CHEK2 kinase. ATM/CHEK2 is activated by DNA damage before phosphorylating multiple proteins, including BRCA1 [64–66]. BRCA1 is phosphorylated at tyrosine residues by ATM, the gene mutated in ataxia telangiectasia, or by ATM and Rad-3-related (ATR) kinase in response to ionizing radiation-induced DNA damage [65,67]. In addition to ATM and ATR, BRCA1 is also phosphorylated by CHEK2, the human homolog of yeast checkpoint protein kinase (hCds1), in response to ionizing radiation-induced DNA damage [66]. This

phosphorylation occurs in a region that contains clusters of serine–glutamine residues, and is functionally important as shown by mouse studies with mutated *BRCA1* that lack these phosphorylation sites. These mice fail to rescue the radiation hypersensitivity when introduced into BRCA1-deficient cells [65]. In addition, BRCA1 phosphorylation by ATM/CHEK2 following DNA damage is critical for recruiting BRCA1 to the DNA repair and chromatin remodeling protein complexes [68].

The potential role of BRCA1 in radioresistant breast cancer has been examined. Studies using breast cancer cell lines report an increased sensitivity to ionizing radiation when *BRCA1* is mutated [13–17]. However, studies of breast cancer patients fail to reliably replicate these *in vivo* findings [69–71]. A recent study found that *BRCA1* mutation carriers exhibit increased sensitivity to radiation therapy by examining the rate of breast cancer recurrence following breast-conserving treatment [72]. However, Pierce and colleagues conducted a multicenter study of *BRCA1* mutation carriers and noted no significant difference between *BRCA1* mutation carriers and women with sporadic breast cancer, in terms of local recurrences [73]. Two additional studies indicated that mutations in *BRCA1* may not account for clinical radiation hypersensitivity [69,71]. These conflicting findings pose the question of whether *BRCA1* mutations will indeed increase the sensitivities of tumor cells to radiation-based therapies. Therefore, the role of BRCA1 and its influence on tumor cell sensitivity to radiation *in vivo* and *in vitro* will require further investigation.

Proteins in BRCA1-associated complexes

Mutations in *BRCA1* may not be the only way to inhibit its activity and, thus, increase cancer susceptibility and sensitivity to radiation-based therapies. There is growing evidence suggesting that disruption of the BRCA1-associated complex, either through mutations or aberrant expression of a key member or members of these multiprotein complexes, may result in loss of normal activity. Significant changes in the stoichiometry of proteins within these complexes may lead to their inactivation, which, in turn, would result in a BRCA1 null-like phenotype. In addition, proteins both upstream of BRCA1, such as ATM, ATR and CHEK2, and downstream, such as p53, RB and CHEK1, when altered, may prevent appropriate activation of BRCA1 or transmission of signals initiated by BRCA1 functional complexes, respectively (FIGURE 2). This may help to explain why BRCA1 itself is not frequently mutated in sporadic disease. BRCA1-associated proteins may, therefore, become potential targets for the treatment of breast cancer, including radiation therapy. A number of studies have reported that manipulation of BRCA1-associated proteins has an impact on cellular resistance or sensitivity to ionizing radiation (TABLE 1) [70,74–83].

In this aspect, a novel multiprotein complex, termed BRCC, containing seven polypeptides, including BRCA1, BRCA2, BARD1 and RAD51, has recently been identified [50]. BRCC is an ubiquitin E3 ligase complex that can cause E2-dependent ubiquitination of the tumor suppressor, p53. In this multiprotein complex, one protein, referred to as BRCC36, is associated with BRCA1 and BRCA2 and plays an important role in the regulation of the ubiquitin E3 ligase activity of BRCC. BRCC36 displays sequence homology with the human Poh1/Pad1 subunit of the 26S proteasome, and with subunit five (Jab1) of the COP9 signalosome. Cancer-associated mutations in *BRCA1* abrogate the association of BRCC36 with BRCC. Reconstitution of a recombinant four-subunit BRCC complex, BRCA1/BARD1/BRCC45/BRCC36, revealed an enhanced E3 ligase activity compared with that of the BRCA1/BARD1 heterodimer [50]. In addition, the authors have reported a profound increase in *BRCC36* expression in the majority of breast cancer cell lines and clinical breast tumors [50]. Importantly, depletion of BRCC36 enhances apoptosis in breast cancer cells following ionizing radiation by preventing the

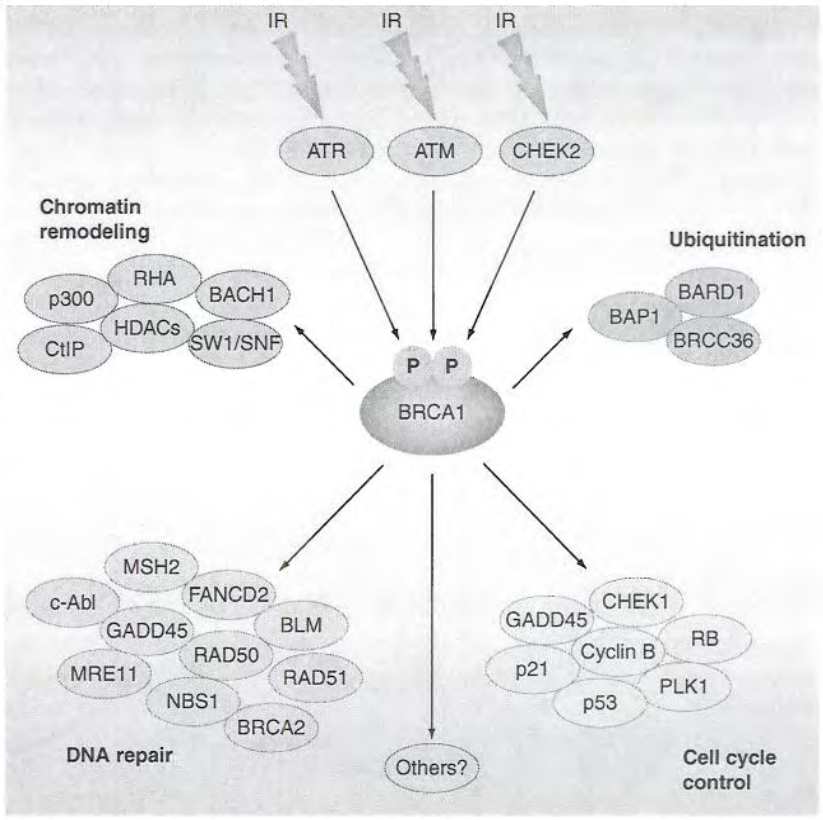

Figure 2. BRCA1-associated protein network. BRCA1 interacts with a number of proteins to form multisubunit protein complexes. BRCA1-associated protein complexes are involved in DNA repair, cell cycle checkpoint control, protein ubiquitination and chromatin remodeling.
BARD1: BRCA1-associated RING domain; IR: Ionizing radiation; BRCA1: Breast cancer susceptibility gene 1.

Table 1. Radiation sensitivity studies related to the BRCA1-associated proteins.

Protein name	BRCA1 pathway	Manipulation approach	Increase resistance	Increase sensitivity
FANCD2	DNA repair	Defected		Garcia-Higuera et al., 2001 Houghtaling et al., 2005
NBS1	DNA repair	Defected		Nakanishi et al., 2002
MRE11	DNA repair	Disrupted		Digweed et al., 2002
RAD51	DNA repair	Deficiency Blocking Overexpression	 Vispe et al., 1998	Lio et al., 2004 Russell et al., 2003
HDAC	Chromatin remodeling	Blocking		Chinnaivan et al., 2005
RB	Check point control	Decreasing Defected		Carlson et al., 2000 Billecke et al., 2002
BARD1	Ubiquitination	Depleted		Boulton et al., 2004
BRCC36	Ubiquitination	Depleted		Dong et al., 2003

BARD: BRCA1-associated RING domain; BRCC: BRCA1 and BRCA2 containing complex.

phosphorylation (i.e., activation) of BRCA1 and by disrupting the BRCA1 nuclear foci formation following IR [CHEN ET AL., UNPUBLISHED OBSERVATIONS]. Based on the role of BRCA1 in DNA repair, the authors propose that disrupting activation of BRCA1 by BRCC36 depletion will create an imbalance between the DNA repair/cell survival and cell apoptosis/death pathways in cells following IR exposure (FIGURE 3). As a result, BRCC36 depletion is likely to substantially sensitize breast tumor cells to IR-induced apoptosis. Due to the fact that the BRCC36 protein has been found aberrantly expressed in the majority of breast cancers and is a major regulator of BRCA1 function, it may serve as a therapeutic target in the management of radiation-resistant breast tumors. Since these findings are mainly based on *in vitro* studies, further examination of the *in vivo* influence of these protein targets on cell sensitivity to radiation is warranted.

Expert commentary

In the last decade since *BRCA1* was cloned, significant progress has been made in defining its biochemical and biological functions and its role in breast and ovarian cancers. Mutations in *BRCA1* have been established to predispose women to breast and ovarian cancer. The BRCA1 protein is also involved in the cellular processes of DNA repair, gene transcription, ubiquitination and chromatin modification. However, little is known about the mechanism by which BRCA1 modulates these processes. Extensive studies have shown that BRCA1 interacts with a number of regulatory proteins to form functional complexes. The activation or inactivation of these BRCA1-associated

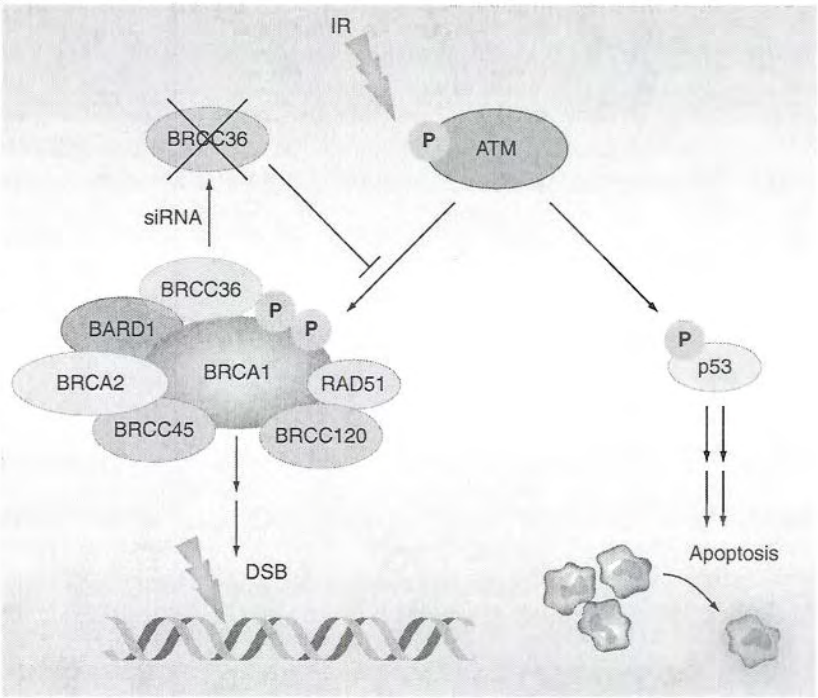

Figure 3. A proposed model illustrating the role of BRCC36 in the BRCA1-associated DNA repair pathway in response to ionizing radiation. BRCA1 and p53 are phosphorylated by ATM induced by IR, and are involved in DNA repair and apoptosis, respectively. Depletion of BRCC36 prevents the phosphorylation of BRCA1 and disrupts the BRCA1 nuclear foci formation in breast cancer cells following ionizing radiation. Based on the role of BRCA1 in DNA repair, the authors propose that disrupting activation of BRCA1 by BRCC36 depletion will create an imbalance between the DNA repair/cell survival and cell apoptosis/death pathways in cells, following exposure to ionizing radiation. As a result, abrogation of BRCC36 appears to sensitize breast cancer cells to IR-induced apoptosis.
ATM: Ataxia telangiectasia-mutated gene; BARD: BRCA1-associated RING domain; BRCC: BRCA1 and BRCA2 containing complex; DSB: Double strand break; IR: Ionizing radiation.

proteins may modify both the risk of developing cancers in *BRCA1* mutation carriers and the efficacy of breast cancer therapy, including radiation therapy.

Five-year view

Further efforts are needed to clarify, in detail, the mechanisms by which BRCA1 collaborates with other key proteins and the molecular pathway through which it is regulated. It will also be helpful to explore the role of other factors that associate with BRCA1, such as BRCA2, RAD51, BARD1, BAP1, BRCC proteins and a potential host of others. These studies may help uncover the range of biological and biochemical functions of BRCA1 and its associated proteins, in order to better elucidate their role in the pathogenesis of breast and ovarian cancer. Only then will it be possible to intelligently design molecular-targeted therapies that may selectively attack these cancer cells.

Acknowledgements

The authors would like to acknowledge the support from an AACR–Anna Barker Award for basic research (to XC), Cheryl Herman and the Eileen Stein-Jacoby Fund, and grants from the National Institutes of Health, Ovarian Cancer SPORE P50 CA83638, and the Department of Defense Breast Cancer Research Program of the US Army Medical Research, DAMD17-03-0312 and DAMD17-03-1-0707 (to AKG) and W81XWH-04-1-0573 (to XC).

Key issues

- Breast cancer susceptibility gene 1 (*BRCA1*) was cloned in 1994 and encodes a 220-kDa nuclear phosphoprotein (1863 amino acids).
- Among thousands of genetic alterations detected in *BRCA1*, more than 700 are deleterious germline mutations that are associated with an increased risk of breast and ovarian cancer.
- The lifetime risk of cancer among female *BRCA1* mutation carriers is estimated to be as high as 80% for breast cancer and more than 40% for ovarian cancer.
- BRCA1 interacts with a number of proteins to form multisubunit protein complexes.
- The number of these protein complexes and their complexity has yet to be fully elucidated.
- BRCA1-associated protein complexes are involved in DNA repair, cell cycle checkpoint control, protein ubiquitination and chromatin remodeling.
- The complete spectrum of the biochemical functions of BRCA1 and its interactors is not fully understood.
- Despite the benefits of radiation therapy in the treatment of breast cancer, patients still develop local recurrences in the targeted breast.
- BRCA1-deficient breast cancer cell lines show increased sensitivity to ionizing radiation. However, studies of breast cancer *BRCA1* carriers fail to reliably replicate these findings *in vivo*.
- A number of studies have reported that manipulation of BRCA1-associated proteins impacts cellular resistance or sensitivity to ionizing radiation.
- Significant changes in the stoichiometry of proteins within these complexes may lead to their inactivation, which, in turn, would result in a BRCA1 null-like phenotype.
- BRCA1-associated proteins may serve as potential therapeutic targets in treating familial and sporadic forms of breast cancer and in enhancing the efficacy of conventional therapies, including radiation-based approaches.

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Website

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Human Molecular Genetics, 2008, Vol. 17, No. 9 ***1336–1348***
doi:10.1093/hmg/ddn022
Advance Access published on January 19, 2008

Allelic imbalance in *BRCA1* and *BRCA2* gene expression is associated with an increased breast cancer risk

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Received October 1, 2007; Revised and Accepted January 16, 2008

The contribution of *BRCA1* and *BRCA2* to familial and non-familial forms of breast cancer has been difficult to accurately estimate because of the myriad of potential genetic and epigenetic mechanisms that can ultimately influence their expression and involvement in cellular activities. As one of these potential mechanisms, we investigated whether allelic imbalance (AI) of *BRCA1* or *BRCA2* expression was associated with an increased risk of developing breast cancer. By developing a quantitative approach utilizing allele-specific real-time PCR, we first evaluated AI caused by nonsense-mediated mRNA decay in patients with frameshift mutations in *BRCA1* and *BRCA2*. We next measured AI for *BRCA1* and *BRCA2* in lymphocytes from three groups: familial breast cancer patients, non-familial breast cancer patients and age-matched cancer-free females. The AI ratios of *BRCA1*, but not *BRCA2*, in the lymphocytes from familial breast cancer patients were found to be significantly increased as compared to cancer-free women (*BRCA1*: 0.424 versus 0.211, $P = 0.00001$; *BRCA2*: 0.206 versus 0.172, $P = 0.38$). Similarly, the AI ratios were greater for *BRCA1* and *BRCA2* in the lymphocytes of non-familial breast cancer cases versus controls (*BRCA1*: 0.353, $P = 0.002$; *BRCA2*: 0.267, $P = 0.03$). Furthermore, the distribution of under-expressed alleles between cancer-free controls and familial cases was significantly different for both *BRCA1* and *BRCA2* gene expression ($P < 0.02$ and $P < 0.02$, respectively). In conclusion, we have found that AI affecting *BRCA1* and to a lesser extent *BRCA2* may contribute to both familial and non-familial forms of breast cancer.

INTRODUCTION

Breast cancer is the most common cancer affecting women, with a lifetime risk among females ~10% by the age of 80 years. In the USA, it has been reported that there will be approximately 180 510 new cases of breast cancer, and more than 40 910 breast cancer-related deaths in 2007 (1). Current estimates suggest that family history is associated with 10–20% of breast cancer (2,3). *BRCA1* (OMIM: 113705) and *BRCA2* (OMIM: 600185) are two of the most prominent breast cancer susceptibility genes and deleterious mutations in these two genes are estimated to account for about 15–30% of familial breast cancer (4–6).

Germline mutations affecting the coding region of *BRCA1* and *BRCA2* are thought to lead to expression of mutant proteins, which are either inactive or function as dominant negatives. However, these scenarios have not been supported by functional studies (7–9). In fact, *Brca1* and *Brca2* knockout mouse models have demonstrated that elimination of Brca1 or Brca2 proteins is sufficient for the development of mammary cancer (10,11). Previously, we have reported that mutant *BRCA1* mRNAs containing premature stop codons were eliminated or destabilized by nonsense-mediated mRNA decay (NMD) (12) and lead to a state of haploinsufficiency. As a result, the ratios between the expressions from the mutant alleles and the corresponding wild-type alleles were

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significantly decreased, resulting in what was referred to as allelic imbalance (AI). AI of *BRCA1* or *BRCA2* expression could decrease the level of both transcripts and proteins and thus contribute to increased susceptibility of developing breast cancer.

There is growing evidence to support this concept. Epigenetic studies have shown that loss of *BRCA1* expression due to promoter hypermethylation is associated with ~10% of sporadic cases of breast and ovarian cancer (13–18). However, screens to evaluate AI have not been applied in depth to study its potential role in the genesis of familial forms of these diseases. A previous study reported that 6 out of 13 human genes, including *BRCA1* and *p53*, were expressed with significant difference between the two alleles, and this difference was transmitted by Mendelian inheritance (19). Furthermore, Yan *et al.* (20) observed that decreased expression of one of the adenomatous polyposis coli tumor suppressor gene (*APC*) alleles was associated with the development of familial adenomatous polyposis. Their studies also found that even more modest decreases in the expression of one *APC* allele could contribute to attenuated forms of polyposis (20). Based on these findings, we hypothesize that a subset of non-*BRCA1/2* mutation carriers with a strong family history of breast cancer are at increased risk of developing this disease as a result of AI in *BRCA1* and *BRCA2* gene expression.

In the present study, we have developed a quantitative approach to measure the allele-specific expression of *BRCA1* and *BRCA2*. We compared *BRCA1/2* allelic variation in a cohort of *BRCA1/2* mutation-negative familial breast cancer patients, non-familial breast cancer patients and age-matched cancer-free volunteers. Since susceptibility to breast cancer is far from being fully understood, our study may help to further identify genetic factors which contribute to breast cancer susceptibility.

RESULTS

Development of a quantitative allelic imbalance assay

In order to determine if allele-specific real-time PCR is able to quantitatively measure the AI in *BRCA1* and *BRCA2* gene expression from the individual allele, RNAs were isolated from the blood lymphocytes of two individuals determined by genotype and sequence analysis to be homozygous for either *BRCA1*-c.4308T/T or *BRCA1*-c.4308C/C (Fig. 1A). This polymorphism was chosen since it is relatively common, based on NCBI dbSNP data. The samples were then reverse transcribed and the cDNAs were mixed at various ratios (8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8) as described in the Materials and Methods section. *BRCA1*-c.4308T/T was detected by the VIC fluorescence signal and *BRCA1*-c.4308C/C was detected by the FAM fluorescence signal. As shown in Figure 1B, with decreasing cDNA ratios of c.4308T to c.4308C, the VIC curve (detecting the c.4308T allele) shifted to the right with the increasing value of $C_{T\text{-c.4308T (VIC)}}$, while the curve of FAM (detecting c.4308C allele) shifted to the left with the decreasing value of $C_{T\text{-c.4308C (VIC)}}$. At the same time, the value of ΔC_T ($C_{T\text{-c.4308T (VIC)}} - C_{T\text{-c.4308C (FAM)}}$) changed from the negative to the positive. By the regression analysis, a linear relationship between Log_2 ratio of cDNAs c.4308T to c.4308C and ΔC_T was identified: Log_2 (c.4308T/C) = $-0.0877 + 1.57897 * \Delta C_T$ ($P < 0.001$) (Fig. 1C). The Pearson correlation coefficient (r) between Log_2 (c.4308T/c.4308C) and ΔC_T was 0.9798. To establish a similar standard curve for *BRCA2* allelic expression, cDNAs from two individuals, who were either homozygous for *BRCA2*-c.3396A/A or *BRCA2*-c.3396G/G, were mixed at the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8 (c.3396A/A allele:c.3396G/G allele). *BRCA2*-c.3396A was detected by the VIC fluorescence signal and *BRCA2*-c.3396G was detected by the FAM fluorescence signal. As shown in Figure 1D, with decreasing ratios of c.3396A to c.3396G, the VIC curve (detecting c.3396A allele) shifted to the right while the FAM curve (detecting c.3396G allele) shifted to the left. After regression analysis, a linear relationship between Log_2 (c.3396A/c.3396G) and ΔC_T was identified: Log_2 (c.3396A/G) = $0.11726 + 1.26458 * \Delta C_T$ ($P < 0.001$) (Fig. 1E). The Pearson correlation coefficient (r) between Log_2 (c.3396A/G) and ΔC_T was 0.9868.

Detection of allelic imbalance caused by nonsense-mediated mRNA decay

To examine whether the allele-specific real-time PCR assay is able to detect AI of *BRCA1* and *BRCA2* gene expression in cell lines, we evaluated RNAs isolated from lymphoblastoid cell lines (LCLs) which were derived from deleterious mutation carriers heterozygous for *BRCA1*-c.3671ins4 or *BRCA2*-c.796delT. These frame-shift mutations create the premature stop codons, which are predicted to activate the NMD pathway and thus lead to decreased levels of mRNAs from the mutant alleles (12). As shown in Figure 2A and B, the ratios of *BRCA1*-c.4308T to -c.4308C between wild type and *BRCA1*-c.3671ins4 heterozygous samples were 0.93 ± 0.04 and 2.07 ± 0.06, respectively ($P < 0.01$). By subcloning and sequencing the individual transcripts, we found that the under-expressed allele contained both the *BRCA1*-c.3671ins4 mutation and the *BRCA1*-c.4308C polymorphism (detected by the FAM signal) (data not shown). To further examine if the loss of *BRCA1*-c.3671ins4 was associated with NMD, we treated the *BRCA1*-c.3671ins4 LCLs with puromycin, a translational inhibitor, 14 h prior to RNA isolation. The ratio of *BRCA1*-c.4308T to -c.4308C in *BRCA1*-c.3671ins4 heterozygous cells decreased ~30%, in comparison to the non-treatment group (1.50 ± 0.05 versus 2.07 ± 0.06, $P < 0.01$) (Fig. 2B). Our data indicated that treatment with puromycin was able to partially recover the AI caused by NMD. Significant AI was also observed for the *BRCA2*-c.796delT mutant allele. The ratios of *BRCA2*-c.3396G to -c.3396A between wild-type and *BRCA2*-c.796delT heterozygous samples were 0.98 ± 0.06 and 6.59 ± 1.31, respectively ($P < 0.01$). After treating the *BRCA2*-c.796delT LCLs with puromycin, the ratio of *BRCA2*-c.3396G to -c.3396A in *BRCA2*-c.796delT heterozygous cells decreased ~31%, in comparison to the non-treatment group (4.90 ± 0.87 versus 6.25 ± 1.17) (Fig. 2C and D). Our results suggested that the loss of expression of *BRCA1* or *BRCA2* mutant alleles via NMD significantly contributed to the observed AI.

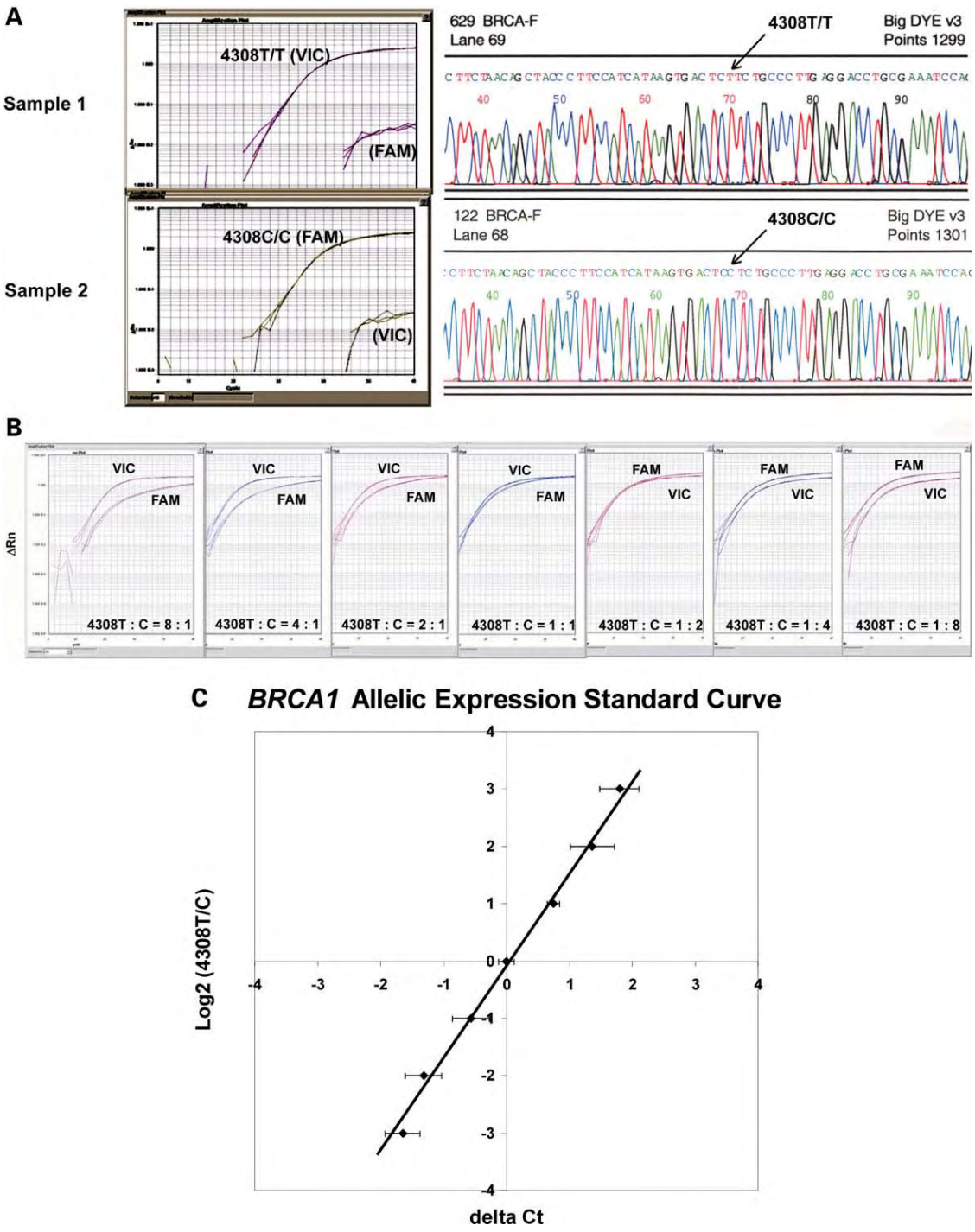


Figure 1. Standard curves for *BRCA1* and *BRCA2* allelic imbalance.
(**A**) Allele-specific real-time PCR amplification plot analyses of *BRCA1*-c.4308T (VIC) and -c.4308C (FAM) was performed in cDNAs generated by RT–PCR using RNAs from blood lymphocytes of two individuals homozygous for either the *BRCA1*-c.4308T/T or *BRCA1*-c.4308C/C. DNA sequencing chromatograms confirming the genotype are shown in the right panel. (**B**) Allele-specific real-time PCR amplification plot was analyzed in mixed cDNAs of *BRCA1*-c.4308T/T (detected by VIC) and *BRCA1*-c.4308C/C (detected by FAM) at the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8, respectively. (**C**) The standard curve for *BRCA1* allelic imbalance: Log_2 (c.4308T/C) = −0.0877 + 1.57897 * ΔC_T. The Pearson correlation coefficient (*r*) between Log_2 (c.4308T/c.4308C) and ΔC_T was 0.9798 (Data expressed as Mean ± SD, *n*=3; the mean value of ΔC_T for c.4308T/C=1 has been adjusted to zero). (**D**) Allele-specific real-time PCR amplification plot was analyzed in mixed cDNAs of *BRCA2*-c.3396A/A (detected by VIC) and *BRCA2*-c.3396G/G (detected by FAM) at the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8, respectively. (**E**) The standard curve for *BRCA2* allelic imbalance: Log_2 (c.3396A/G) = 0.11726 + 1.26458 * ΔC_T. The Pearson correlation coefficient (*r*) between Log_2 (c.3396A/G) and ΔC_T was 0.9868 (Data expressed as Mean ± SD, *n* = 3; the mean value of ΔC_T for c.3396A/G = 1 has been adjusted to zero).

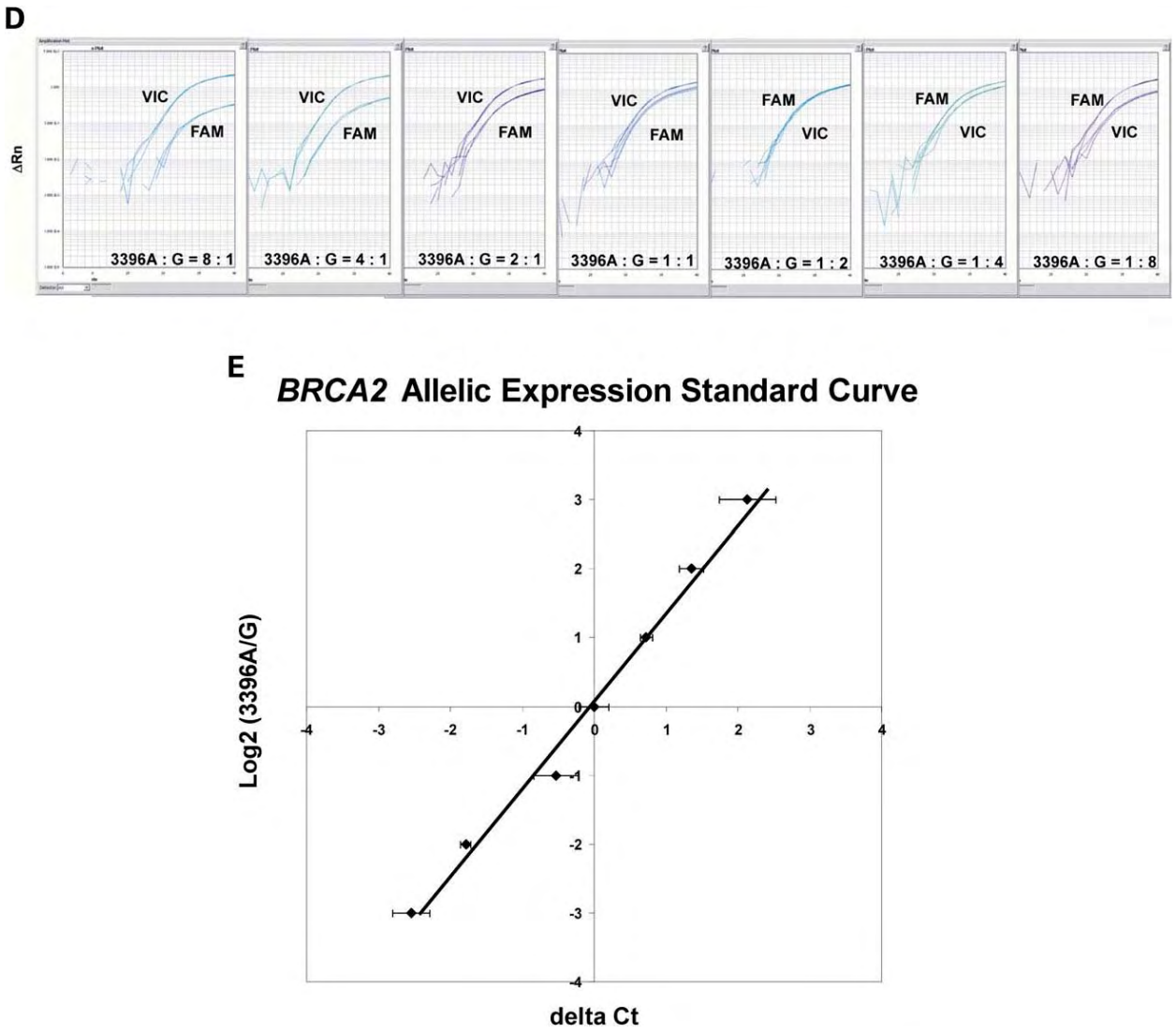
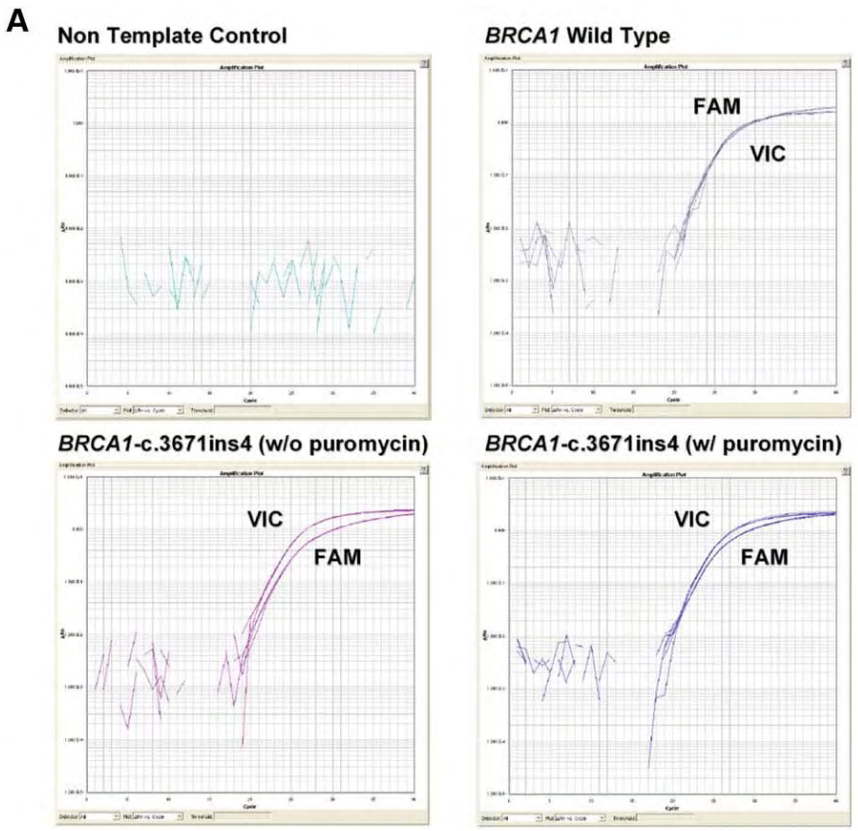


Figure 1. *continued*

BRCA1 and *BRCA2* allelic imbalance is associated with breast cancer risk

To evaluate AI of *BRCA1* and *BRCA2* gene expression, genotype analysis of the two common polymorphisms, *BRCA1*-c.4308T/C and *BRCA2*-c.3396A/G, was performed on DNA samples isolated from fresh-frozen peripheral blood lymphocytes from 85 unrelated *BRCA1/2* mutation-negative familial breast cancer carriers (median age at sample collection: 47), 112 non-familial breast cancer carriers (median age at sample collection: 52) and 102 age-matched cancer-free females (median age at sample collection: 51) (Table 1). From these analyses, 37 (43.5%), 48 (42.9%) and 41 (40.2%) of the samples evaluated were determined to be heterozygote for the *BRCA1*-c.4308T/C polymorphism for familial breast cancer patients, non-familial cancer patients and cancer-free controls, respectively (Table 1). Furthermore, 39 (45.9%), 44 (39.3%) and 36 (35.3%) of the samples above were found to be heterozygous for the *BRCA2*-c.3396A/G polymorphism (Table 1). Since our initial validation studies were preformed using immortalized LCLs, we first compared AI in RNA isolated from 20 fresh-frozen lymphocytes versus 20 established Epstein-Barr Virus (EBV)-lines. No significant differences were detected between these two sample sets [*BRCA1*: 0.424 ± 0.129 versus 0.409 ± 0.127 ($n = 11$); *BRCA2*: 0.212 ± 0.180 versus 0.225 ± 0.209 ($n = 10$)]. However, to limit any AI variation potentially introduced by EBV transformation, all subsequent AI assays were performed using RNAs isolated from peripheral blood lymphocytes. Next, RNA isolated from *BRCA1*-c.4308T/C ($n = 126$) and *BRCA2*-c.3396A/G ($n = 119$) heterozygotes, including single heterozygotes and double heterozygotes, were evaluated for integrity and quantity. Those samples demonstrating high quality and the necessary quantities were used in the AI assay, as described in the Materials and Methods section.

To evaluate the AI, we used the absolute values of Log_2 (*BRCA1*-c.4308T/C) or Log_2 (*BRCA2*-c.3396A/c.3396G).



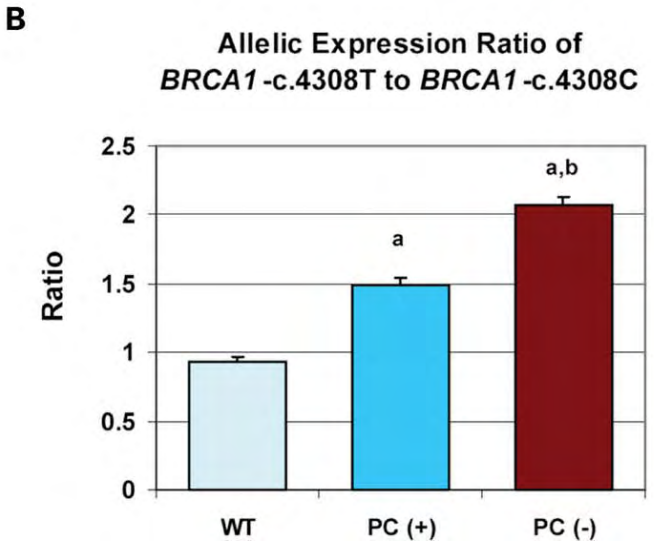


Figure 2. *BRCA1* and *BRCA2* allelic imbalance caused by NMD. (**A**) Allele-specific real-time PCR amplification plots of *BRCA1*-c.4308T (VIC) and -c.4308C (FAM) for non-template control, *BRCA1* wild-type lymphoblastoid cells (WT), *BRCA1* mutant (heterozygous *BRCA1*-c.3671ins4) lymphoblastoid cells without [PC (−)] or with [PC (+)] puromycin treatment. (**B**) Allelic expression ratios of *BRCA1*-c.4308T to *BRCA1*-c.4308C (a: versus WT; b: versus PC (+); *t*-test, $P < 0.05$). (**C**) Allele-specific real-time PCR amplification plots of *BRCA2*-c.3396A (VIC) and -c.3396G (FAM) for non-template control, *BRCA2* wild-type lymphoblastoid cells (WT), *BRCA2* mutant (heterozygous *BRCA2*-c.796delT) lymphoblastoid cells without [PC (−)] or with [PC (+)] puromycin treatment. (**D**) Allelic expression ratios of *BRCA2*-c.3396G to *BRCA2*-c.3396A (a: versus WT; b: versus PC (+); *t*-test, $P < 0.05$).

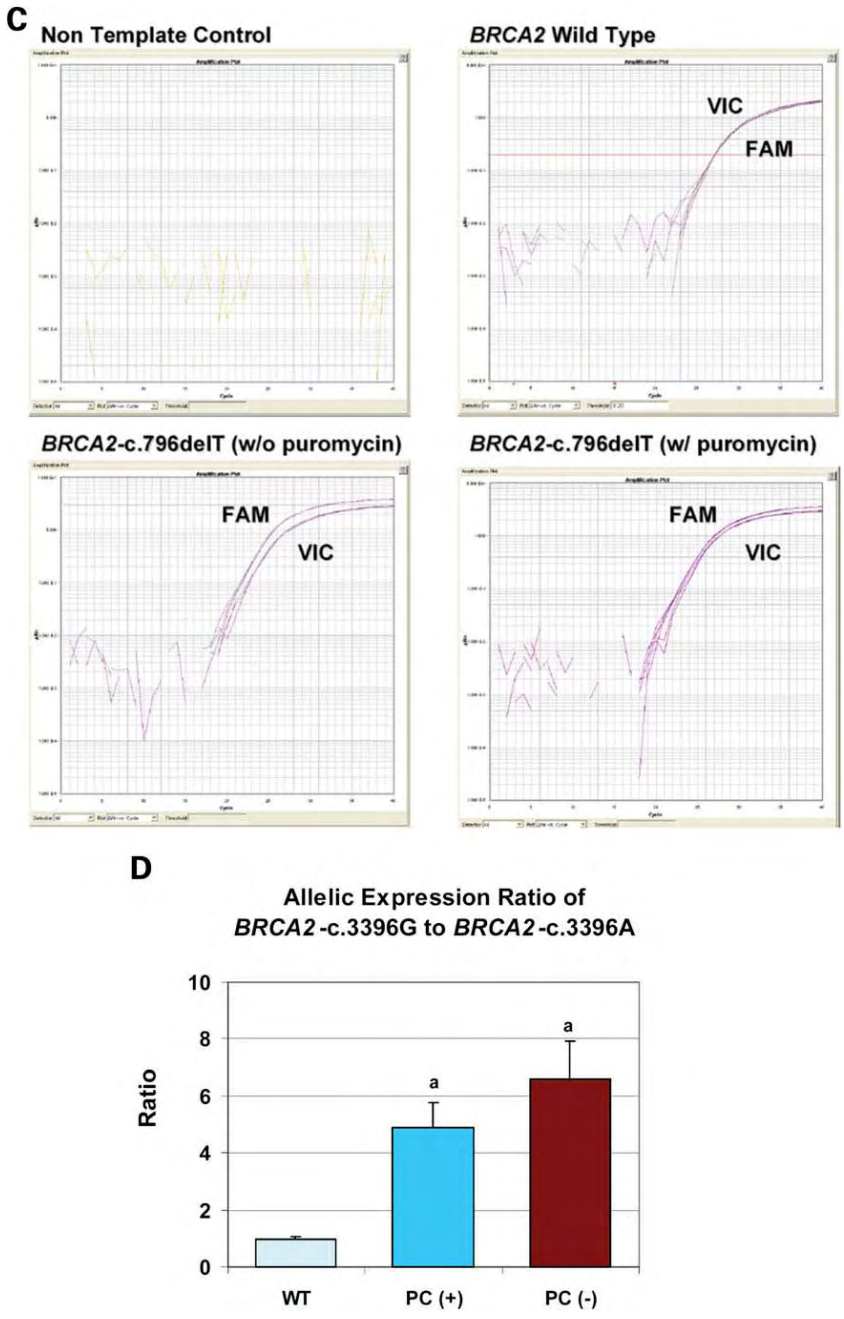


Figure 2. *continued*

The mean value of Log_2 (c.4308T/C) of *BRCA1* in the lymphocytes from familial breast cancer carriers was found to be ~104% higher than that that in the lymphocytes from cancer-free controls [0.424 ± 0.157 ($n = 32$) versus 0.211 ± 0.169 ($n = 40$), $P = 0.00001$; *t*-test] (Table 2 and Fig. 3A and B). Log_2 of *BRCA1*-c.4308T/C in the lymphocytes from non-familial breast cancer carriers was 73% higher than that in cancer-free controls [0.353 ± 0.209 ($n = 32$), $P = 0.002$ versus control] (Table 2 and Fig. 3A and C). In comparison, the mean value of Log_2 of *BRCA2*-c.3396A/G in the lymphocytes from familial breast cancer patients was moderately higher (10%) than that in cancer-free controls [0.206 ± 0.180 ($n = 37$) versus 0.172 ± 0.123 ($n = 31$), $P = 0.38$; *t*-test] (Table 2 and Fig. 4A and B). A similar result (38% higher) was observed for Log_2 (c.3396A/G) of *BRCA2* in the lymphocytes of non-familial breast cancer carriers [0.267 ± 0.171 ($n = 26$), $P = 0.03$ versus control] (Table 2 and Fig. 4A and C).

Interestingly, the distribution of under-expressed alleles of *BRCA1* and *BRCA2* was found to be significantly different between cancer-free control and familial breast carriers, but not between cancer-free control and non-familial breast carriers. As shown in Table 3 and Figure 3, under-expressed

Table 1. Characteristics of the study groups

Characters	Study groups		
	Familial	Non-familial	Cancer-free
Sample size	85	112	102
Age (median)			
At diagnosis	44	49	NA
At sample collection	47	52	51
Family history[a]			
2 or more	85	0	0
1	0	23	25
0	0	89	77
Genotypes			
BRCA1-c.4308T/C	37	48	41
BRCA2-c.3396A/G	39	44	36

[a]Number of first and/or second-degree relatives affected with either breast and/or ovarian cancer.

BRCA1-c.4308T (i.e. Log_2 [4308T/C] < 0) and *BRCA1*-c.4308C (i.e. Log_2 [4308T/C] > 0) alleles were found in ~53% (21 of 40) and ~47% (19 of 40) of cancer-free controls as compared to ~28% (9 of 32) and ~72% (23 of 32) of familial breast cancer carriers, respectively ($P < 0.02$). In addition, under-expressed *BRCA2*-c.3396A [i.e. Log_2 (3396A/G) < 0] and *BRCA2*-c.3396G [i.e. Log_2 (3396A/G) > 0] alleles were found in ~45% (14 of 31) and ~55% (17 of 31) of cancer-free controls as compared to ~70% (26 of 37) and ~30% (11 of 37) of familial breast cancer carriers ($P < 0.02$), respectively (Table 3 and Fig. 4).

Inheritance effects of AI in *BRCA1*

A previous study has indicated that AI for several tumor suppressor genes could be transmitted by Mendelian inheritance (19). To test if the AI observed in our study may be inherited, we identified three affected women (i.e. probands) reporting a significant family history of breast and/or ovarian cancer for which we had blood from at least one of their sisters. Furthermore, each sister had to be heterozygous for the *BRCA1*-c.4308T/C polymorphism. As shown in family A (Table 4), sister Sis-02 displayed a similar AI pattern as compared to the proband, while sister Sis-01 displayed no AI. In the two other families, both the affected probands and their corresponding sisters showed AI (Table 4). We further performed a haplotype analysis to determine whether the alleles showing AI were shared between siblings. As shown in Table 4, sisters with the same AI phenotype shared the same haplotype with their affected sister. Importantly, sister Sis-01 in family A did not share the same haplotype. Her blood sample displayed no AI (0.007 ± 0.147) for *BRCA1* gene expressions whereas the AI was detected in her unaffected and affected sisters (Sis-02 and Proband, 0.382 ± 0.176 and 0.375 ± 0.06, respectively) (Table 4). The allele frequencies of the microsatellite markers used for haplotype construction are listed in Supplementary Material, Table S1.

DISCUSSION

In this study, we developed a quantitative AI assay to examine the expression difference between the alleles of *BRCA1* and *BRCA2* (Fig. 1). By performing this AI assay with specific primers and probes that target common single nucleotide polymorphisms in *BRCA1* and in *BRCA2*, we were able to detect allelic imbalance associated with NMD in patients carrying frameshift mutations in *BRCA1* and *BRCA2* (Fig. 2). We next compared AI of *BRCA1* and *BRCA2* expression among three groups, familial breast cancer patients, non-familial breast cancer patients, and age-matched cancer-free females. AI ratios of *BRCA1* in familial breast cancer cases were significantly higher than those from cancer-free controls ($P = 0.00001$) (Table 2 and Fig. 3). Similar results were observed for AI ratios of *BRCA1* in the lymphocytes from non-familial breast cancer patients ($P=0.002$). AI ratios of *BRCA2* in familial or non-familial breast cancer cases were also higher than those from cancer-free controls ($P = 0.38$ or $P = 0.03$, respectively). However, the difference was not statistically significant in the ratios of mRNA expressed from the *BRCA2* alleles found in familial breast cancer cases when compared to cancer-free controls (Table 2 and Fig. 4). In addition, the distribution of under-expressed alleles between cancer-free controls and familial cases was significantly different for both *BRCA1* and *BRCA2* gene expression ($P < 0.02$ and $P < 0.02$, respectively) (Table 3). Furthermore, we have demonstrated that the AI patterns for *BRCA1* expression, albeit in a small number of families, can be transmitted by Mendelian inheritance (Table 4). Although these findings are consistent with a previous study (19), future evaluations will benefit from evaluating AI in large families for evidence of disease segregation.

Several methods have been developed to evaluate allele-specific expression. The first method combines primer extension and capillary electrophoresis (19,21). The second approach utilizes microarray technology to measure allele-specific mRNA expression (22). Compared to the AI assay presented here, the method of primer extension plus capillary electrophoresis is also accurate but relatively time-consuming and expensive. The microarray approach provides a high-throughput and a powerful platform for the simultaneous analysis of large numbers of genes to analyze allele-specific gene expression, but it has less power to define the AI. Like the majority of allelic expression methods (23), our AI assay also requires a transcribed heterozygous variant in the individuals to be evaluated. In the present study, we targeted two common polymorphisms, *BRCA1*-c.4308T>C and *BRCA2*-c.3396A>G in the general population. Therefore, a substantial number of subjects homozygous for the polymorphisms had to be excluded. To overcome this limitation of population selection based on genotypes, other primers and probes will need to be developed to target other common polymorphisms in *BRCA1* and/or *BRCA2*. In addition, our approach could easily be applied for studying AI in other cancer susceptibility genes, such as *p53*, *APC*, *PTEN,* etc.

In this study, we have demonstrated AI for both *BRCA1* and *BRCA2* in breast cancer populations. Interestingly, the increase of AI ratios in familial and non-familial breast cancer patients was more significant for *BRCA1* than *BRCA2*. Loss of *BRCA1* expression in breast cancer has been reported to be related to the pathogenesis of breast cancer (13–17). Loss of *BRCA2* expression in cancers, in

Table 2. Allelic imbalance in *BRCA1* and *BRCA2* expression

Genes	Population	Sample number	AI (Mean ± SD)[a]	*t*-test (*P*-value) versus cancer-free
BRCA1	Cancer-free	40	0.211 ± 0.169	
	Familial	32	0.424 ± 0.157	0.00001
	Non-familial	32	0.353 ± 0.209	0.002
BRCA2	Cancer-free	31	0.172 ± 0.123	
	Familial	37	0.206 ± 0.180	0.38
	Non-familial	26	0.267 ± 0.171	0.03

[a] To calculate the mean value of AI, all negative value of Log_2 (*BRCA1*-c.4308T/C) and Log_2 (*BRCA2*-c.3396A/c.3396G) in Figures 3 and 4 were changed to positive values.

contrast, is still controversial (24,25). These findings indicate that AI in *BRCA1* appears to be a more common event in breast cancer development than AI involving *BRCA2*. However, the mechanism(s) leading to the observed AI is for the most part unknown.

We have demonstrated that both *BRCA1* and *BRCA2* deleterious mutations can activate the NMD pathway and result in AI [Figure 2, and (12)]. However, all the familial breast cancer patients evaluated in the current study were determined to lack a mutation in *BRCA1* and *BRCA2* that would trigger NMD. Furthermore, we evaluated the *BRCA1* and *BRCA2* genes in the sporadic breast cancer patients and cancer-free controls demonstrating AI [i.e. allele expression ratio > 0.25 or < -0.25 (Figs 3 and 4)]. Again, no deleterious germline mutations were detected (data not shown). This is not entirely surprising given that germline mutations in *BRCA1* and *BRCA2* are rare in women affected with breast cancer without a strong family history of the disease (26–29).

Based on these observations, we conclude that NMD is not likely to be responsible for the observed AI in our case–control comparisons. Therefore, other mechanisms are likely to exist to account for the observed increased AI of *BRCA1* and *BRCA2* gene expression in female breast cancer patients. For example, the 5′ and 3′ non-coding regions of *BRCA1* and *BRCA2* are rarely evaluated through genetic testing, even though genetic alterations in these non-coding regions could be important in regulating *BRCA1* and *BRCA2* expression. For instance, genetic alterations within 5′ DNA or the putative promoter regions are able to disrupt the binding of transcription factors to DNA regulatory elements and hence lead to the loss of allelic gene expression. Several studies have shown that large genomic deletions involving the *BRCA1* promoter were associated with hereditary breast cancer (30–32). This concept is further supported by studies of Cowden syndrome (CS) showing that ∼10% of CS-related *PTEN* mutations occur in the *PTEN* promoter and lead to a 50% reduction in PTEN expression (33,34). Also, allele-specific hypermethylation of the *BRCA1* promoter region and decreased *BRCA1* expression is associated with ∼10% of sporadic breast cancer cases (18,30,35). Recent advances have identified a new pathway for gene regulation, i.e. via microRNAs (miRNAs) (36,37). These 21–22 nt RNA molecules are complementary to the 3′-UTR sequence of transcripts and mediate negative post-transcriptional regulation through RNA duplex formation (36,38). By performing *in silico* analyses in four *BRCA1* SNPs and two *BRCA2* SNPs (39), we have identified three rare *BRCA1* alleles (c.5628G, c.6273T, c.6924A) that could potentially create target sites for selected microRNAs (Supplementary Material, Table S2). Therefore, it is possible that altered mRNA targeting could contribute to AI of *BRCA1* gene expression in the absence of frameshift mutations. It will be important in future studies to determine the mechanisms that either disrupt transcription factors binding or alter miRNA binding, leading to constitutively decreased levels of *BRCA1* and *BRCA2* and an increased risk of developing breast cancer.

In summary, we have developed a quantitative approach to evaluate expression of *BRCA1* and *BRCA2* from individual alleles, and we have found that AI in *BRCA1* and to a lesser extent *BRCA2* is associated with increased breast cancer risk. Furthermore, we have demonstrated that the AI patterns for *BRCA1* expression could be transmitted by Mendelian inheritance. Since susceptibility to breast cancer is far from being fully understood, our study suggests that alternate mechanisms, other than deleterious coding mutations, may contribute to breast cancer.

MATERIALS AND METHODS

Databases

RefSeqs (GenBank Accession No: NM_007295.2 and NM_000059.1) were used for *BRCA1* and *BRCA2* mRNA numbering, respectively. The A of ATG translation initiation codon is defined as position +1.

Subjects and genotype analysis

Three populations were used in this study, (i) *BRCA1/2* mutation-negative women reporting a personal and family history of breast cancer, i.e. familial; (ii) female breast cancer patients without a significant family history of disease, i.e. non-familial; and (iii) age-matched cancer-free female controls (Table 1). All participants were Caucasian women with European-American ancestry and were from the Delaware Valley, including the greater Philadelphia Metropolitan area in Pennsylvania. For family studies, eligible subjects were women with a personal and family history of cancer (at least two first and/or second-degree relatives affected with either breast and/or ovarian cancer) and were ascertained from the Family Risk Assessment Program (FRAP) at the Fox Chase Cancer Center (FCCC). All relevant institutional

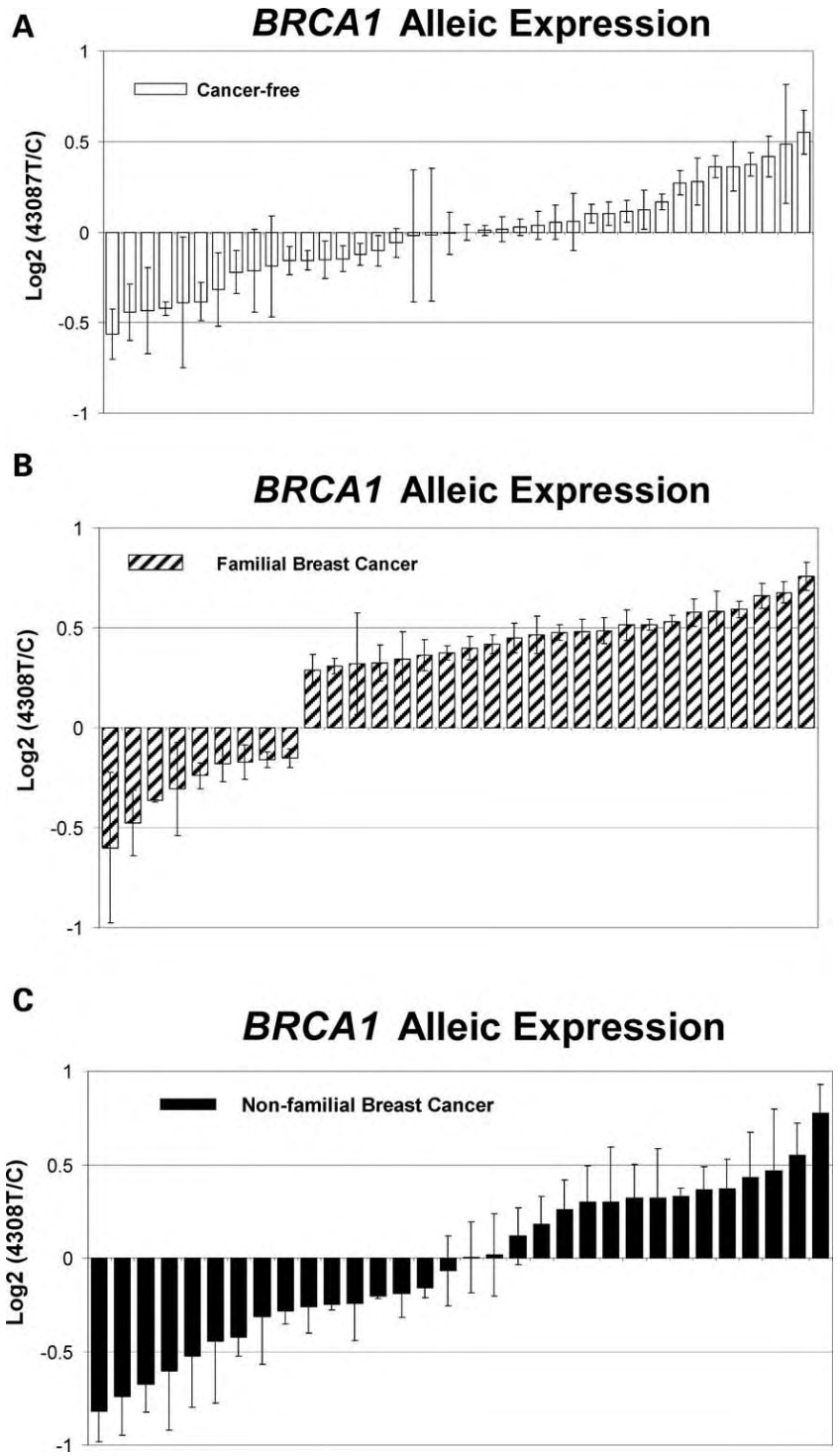


Figure 3. *BRCA1* allelic expression ratios in cancer-free controls, familial and non-familial breast cancer patients. The AI assays were performed using specific primer and probe sets targeting *BRCA1*-c.4308T/C alleles. Log_2 ratios of *BRCA1*-c.4308T allele to -c.4308C allele expression were presented in cancer-free controls (**A**), familial (**B**) and non-familial breast cancer patients (**C**). (Data expressed as Mean $\pm$ SD, $n = 3$; the mean value of allelic expression ratios of total normal samples has been adjusted to zero).

review boards approved the study protocol and written informed consent was obtained from all participants. Genotype analyses of the two common polymorphisms, *BRCA1*-c.4308T/C and *BRCA2*-c.3396A/G were carried out using ABI PRISM 7900HT Sequence Detection System and Assays-on-Demand SNP Genotyping products for fluorogenic polymerase chain reaction allelic discrimination (Applied Biosystems, Foster City, CA, USA).

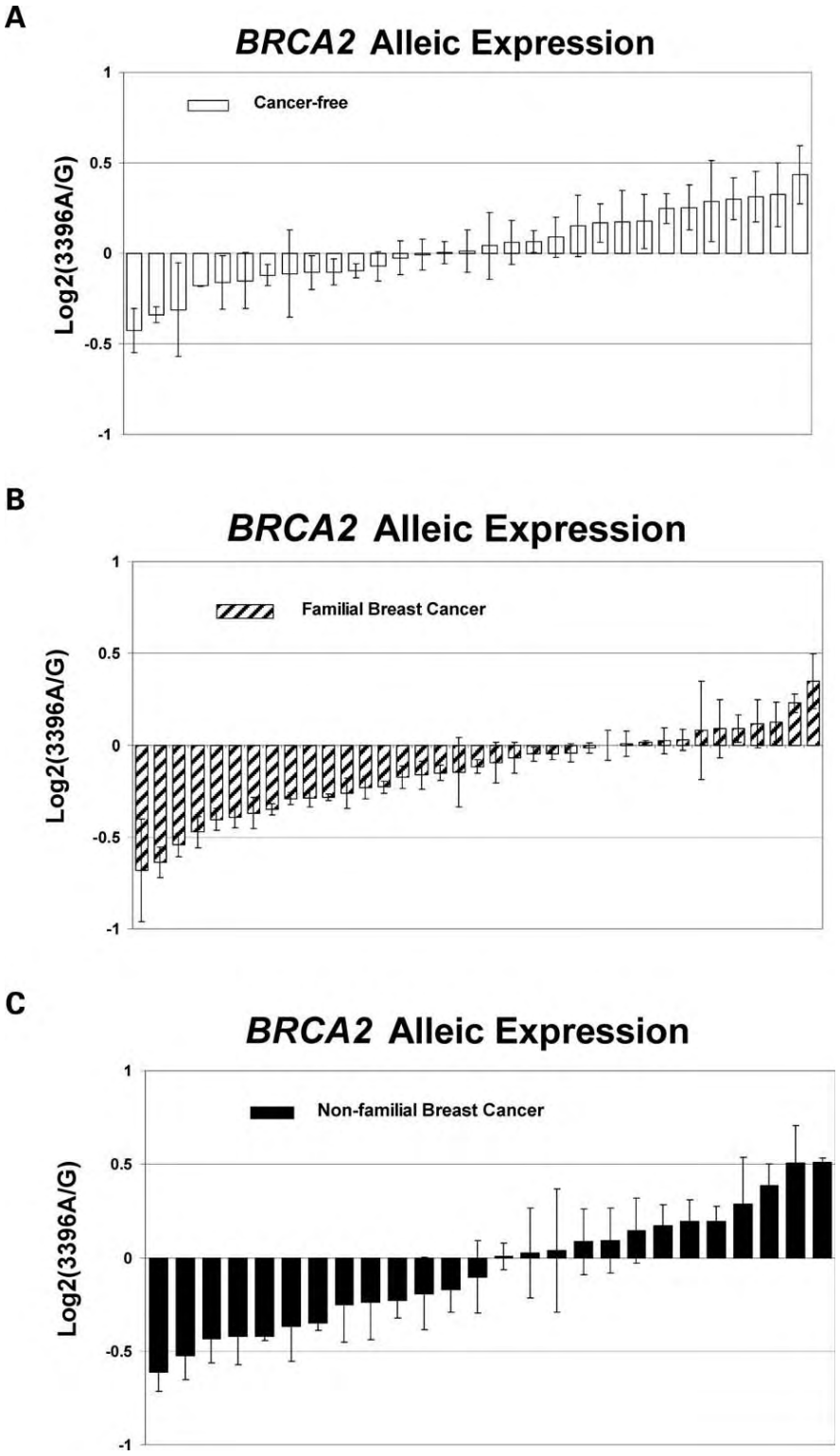


Figure 4. *BRCA2* allelic expression ratios in cancer-free controls, familial and non-familial breast cancer patients. The AI assays were performed using specific primer and probe sets targeting *BRCA2*-c.3396A/G. Log_2 ratios of *BRCA2*-c.3396A allele to -c.3396G allele expression were presented in cancer-free controls (**A**), familial (**B**) and non-familial cancer patients (**C**). (Data expressed as Mean $\pm$ SD, $n = 3$; the mean value of allelic expression ratios of total normal samples has been adjusted to zero).

Table 3. Distribution of under-expressed alleles of *BRCA1* and *BRCA2*

Genes	Group	Under-expressed alleles		OR (95% CI)	*P*-value[a]
BRCA1		c.4308T, Log_2 [4308T/C]<0	c.4308C, Log_2 [4308T/C]>0		
	Cancer-free Controls	21	19		
	Familial	9	23	2.82 (1.05, 7.60)	0.02
	Non-Familial	16	16	1.11 (0.44, 2.80)	0.18
BRCA2		c.3396A, Log_2 [3396A/G]<0	c3396G, Log_2 [3396A/G]>0		
	Cancer-free Controls	14	17		
	Familial	26	11	0.35	0.02
	Non-familial	13	13	0.82	0.20

[a]A χ^2 test was used to assess the 2 by 2 tables.

Table 4. Allelic expression and haplotype analysis of *BRCA1* in sisters from three breast cancer-prone families

Family	Members	Allelic expression [Log_2 (*BRCA1*-c.4308T/C)]	Haplotypes D17S855–D17S1322–D17S1325
Family A	Proband[a]	0.375 ± 0.060	145/155–121/121–193/193
	Sis-01	0.007 ± 0.147	145/153–121/121–195/193
	Sis-02	0.382 ± 0.176	145/155–121/121–193/193
Family B	Proband[b]	0.477 ± 0.070	145/151–121/124–193/193
	Sis-01	0.232 ± 0.214	145/151–121/124–193/193
Family C	Proband[b]	0.583 ± 0.243	145/153–121/127–189/189
	Sis-01	0.522 ± 0.156	145/153–121/127–189/189

[a]Ovarian cancer carrier.
[b]Breast cancer carrier.

Allelic imbalance assay

A 1.25 μl of the cDNA synthesized in the RT reaction was used in a real-time PCR reaction (25 μl total volume), performed with ABI PRISM 7900HT Sequence Detection System following methods recommended by the manufacturer. Optimal conditions were as follows: Step 1, 95°C for 10 min; Step 2, 92°C for 15 s, 60°C for 60 s with Optics; repeated for 40 cycles. The primer and probe sets used in real-time PCR reaction to detected *BRCA1*-c.4308T/C (rs1060915) and *BRCA2*-c.3396A/G (rs1801406) allelic expression were obtained from Applied Biosystem TaqMan® SNP Assay program (Assay ID: C.3178676 and C.7605673.1 for *BRCA1* and *BRCA2*, respectively). Sequence information for primers and probes is available upon request. Each 96-well PCR plate included negative controls, positive controls and unknown samples. Real-time PCR data were analyzed with ABI SDS 2.2.2 software. In order to produce the *BRCA1* allelic expression standard curve, cDNAs from the two samples with homozygous genotypes, *BRCA1*-c.4308T/T and *BRCA2*-c.4308C/C, were mixed as the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8 (c.4308T/T allele:c.4308C/T allele). For the same purpose, cDNAs from the two samples with homozygous genotypes, *BRCA2*-c.3396A/A and *BRCA2*-c.3396G/G, were mixed as the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8 (c.3396A/A allele:c.3396G/G allele).

The principles of quantitative real-time PCR provide the basis of this linear relation between Log_2 ratio and ΔC_T established in our approach to detect AI (40,41). Previous data have shown that AmpliTaq DNA polymerase cleaves the matched and well-hybridized probe and target sequences and produces a fluorescent signal (42). In contrast, mismatches between a probe and target are expected to reduce the efficiency of probe hybridization, and AmpliTaq DNA polymerase is more likely to displace a mismatched probe without cleaving it, which does not produce a fluorescent signal.

Theoretically, Allele 1 gene copy number (detected by FAM):

$$\mathrm{Log}_2[\mathrm{Allele}-1] = -\mathrm{A}_1 * \mathrm{C}_{\mathrm{T}1} + \mathrm{B}_1 \quad (1)$$

and Allele 2 gene copy number (detected by VIC):

$$\mathrm{Log}_2[\mathrm{Allele}-2] = -\mathrm{A}_2 * \mathrm{C}_{\mathrm{T}2} + \mathrm{B}_2 \quad (2)$$

If the fluorescence probes have the same efficiency to hybridize with matched target sequence, that is, $A_1 = A_2 = A$, therefore,

$$\mathrm{Log}_2[\mathrm{Allele}-1/2] = \mathrm{A} * (\mathrm{C}_{\mathrm{T}2} - \mathrm{C}_{\mathrm{T}1}) + (\mathrm{B}_1 - \mathrm{B}_2) \quad (3)$$

The function (3) was confirmed by two standard curves, Log_2 (c.4308T/C) = −0.0877 + 1.57897 * ΔC_T and Log_2 (c.3396A/G) = 0.11726 + 1.26458 * ΔC_T, set up by our experimental data (Fig. 1). Besides using function (3) to calculate the ratio of mRNA expression between the two alleles, function (1) and function (2) are able to be applied for examining the absolute value of each allele mRNA expression. However, the direct analysis of single allele expression is often complicated by the potential variations between individuals with different environmental or physiological background rather than genetic factors. Comparing the relative expression levels of two alleles of the same gene within the same biologic sample will help to minimize these variations.

Peripheral blood lymphocytes and LCLs

Lymphocytes were isolated from peripheral blood and stored at −150°C until needed. None of the blood samples from breast cancer patients were collected at the time of chemo- or radiation therapy. In addition, a subset of cyropreserved lymphocytes from *BRCA1* or *BRCA2* mutation carriers (e.g. *BRCA1*-c.3671ins4 and *BRCA2*-c.796delT) or disease-free individuals were infected with EBV to establish immortal LCLs. LCLs were maintained in RPMI (GIBCO BRL) media supplemented with 20% fetal calf serum and antibiotics at 37°C, 5% CO_2 atmospheric condition and 95% humidity. The immortalized LCLs from cancer-free individuals that had been tested negative for mutations in *BRCA1* and *BRCA2* served as wild-type controls. To prevent potential degradation of unstable transcripts by NMD a translation inhibitor, puromycin (Sigma, St Louis, MO, USA) was added to the LCL cells as described in a previous study (12).

Subcloning the PCR product and sequence analysis

PCR fragments containing a common polymorphism and deleterious mutation were subcloned directly into pCR®4-TOPO vector (Invitrogen, Carlsbad, CA, USA). PCR was then performed to identify bacterial colonies containing appropriate inserts. Plasmid DNA was purified using QIAfilter™ Plasmid Maxi Kit (Qiagen Inc., Valencia, CA, USA) and the insert was sequenced using either the universal M13-primers or the primers for PCR reactions.

RNA isolation and reverse transcription (RT)

Total cellular RNAs were isolated from blood lymphocyte pellets using TRIzol reagent according to the protocols provided by the manufacturer (Invitrogen Corp., Carlsbad, CA, USA). Purified RNAs were further processed to remove any contaminating DNA (DNA-free kit, Ambion, Inc., Houston, TX, USA). After quantification with Bioanalyzer-2100 system using RNA 6000 Nano LabChip kits (Agilent Technologies, Palo Alto, CA, USA), 2 μg of total RNA from each sample was used as a template to be reverse-transcribed (RT) in a 20 μl reaction [containing 5 μM random hexamers, 500 μM deoxynucleoside triphosphate mix, 1× RT (reverse transcriptase) buffer, 5 mM $MgCl_2$, 1.5 units of RNase inhibitor and 7.5 units of MuLV reverse transcriptase]. All reagents were purchased from Applied Biosystems (Branchburg, NJ, USA). The RT reaction conditions were 10 min at 25°C, 1 h at 42°C and 5 min at 94°C.

Haplotype analysis

Haplotypes were constructed for *BRCA1* using three polymorphic microsatellite repeat markers located within (D17S855 and D17S1322) or adjacent (D17S1325) to the *BRCA1* locus. The sequences of the primer pairs were obtained from the Genome Database (http://www.gdb.org) and PCR reaction was carried out as previously reported (43,44). PCR products with fluorescent dye (HEX) labeled primer were mixed with Hi-Di Formamide and a fluorescent labeled internal size marker. The mixture was subjected to electrophoreseis on an ABI 3100 Automated DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and the data were analyzed by the GeneScan (Version 3.7) and GeneMapper (Version 4.0) software provided by the manufacturer.

Statistical analysis

Allele specific real-time PCR data were analyzed with ABI SDS software v2.2.2 (Applied Biosystems, Foster City, CA, USA). Statistical analysis was conducted using the SAS System (version 9) developed by the SAS Institute, Inc. (Cary, NC, USA). Student's *t*-test was employed for continuous data and results were presented as the mean ± SD. We compared the distribution of under-expressed alleles in *BRCA1* or *BRCA2* between cases and controls using χ^2 95% confidence intervals (CI) and the difference in distribution of under-expressed alleles was estimated as odds ratios (OR). A value of $P < 0.05$ is considered significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We would like to acknowledge Dr S. Litwin for help with statistical analyses and Ms K.A. Cattie with haplotype analysis. We also thank the numerous volunteers for providing blood samples for these studies.

Conflict of Interest statement. None of the authors has any conflict of interest.

FUNDING

This work is supported in part by Cheryl Herman and the Eileen Stein-Jacoby Fund, grants from the National Cancer Institute, i.e. P50 CA83638 (A.K.G.), U01 CA69631 (M.B.D.) and R25 CA057708 (S.W.); a fellowship from the Department of Defense, W81XWH-04-1-0573 (X.C.); and DOD grants, DAMD17-03-1-0707 (A.K.G.) and DAMD17-03-1-0312 (A.K.G.); and by an appropriation from the Commonwealth of Pennsylvania.

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